The roles of endogenous Calcium/Calmodulindependent kinase II inhibitors in learning and memory

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The roles of endogenous Calcium/Calmodulin-dependent kinase II inhibitors in learning and memory.

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Thesis submitted for the degree of PhD

2015

“To think is to forget differences, generalize, make abstractions.”

Funes the memorious, in Ficciones by Jorge Luis Borges (1942)

Abstract

Calcium/Calmodulin-dependent kinase II (CaMKII) is a serine/threonine kinase with a wide range of substrates. A number of studies have established that CaMKII is fundamentally important for various learning and memory processes. Given this importance the activity of CaMKII must be tightly regulated. Two endogenous inhibitor proteins of CaMKII, CaMK2N1 and CaMK2N2, have been identified. During contextual fear memory formation CaMK2N1 and CaMK2N2 expression increases in brain regions that are related to the task. However, the functions of CaMK2Ns are still unknown. Our aim was to study the physiological roles of these inhibitors in memory and learning. For that purpose we used adeno-associated virus vector to either knockdown the expression of CaMK2N1 or overexpress CaMK2N2 in the dorsal hippocampus of mice. Animals were trained and tested in contextual fear conditioning paradigm. The knockdown of CaMK2N1 expression had no effect on long-term memory formation, but it impaired long-term memory maintenance after retrieval. Western blot analyses revealed that CaMK2N1 knockdown prevents a decrease in T286 phosphorylation of αCaMKII induced by memory testing, as well as a reduction of GluA1 and c-fos levels. This puts forward the hypothesis that CaMK2N1 is necessary for inducing a decrease in neuronal activation after memory retrieval and that this process is required for memory maintenance. Regarding the hippocampal overexpression of CaMK2N2, treatment prior to training blocked contextual memory formation. On the other hand, overexpression of the same inhibitor after training had no effect on contextual long-term memory maintenance. These results support the view persistent CaMKII activity is not the molecular basis of long-term memory maintenance but its inhibition is.

Acknowledgements

I would like to thank my supervisor Dr. Karl Peter Giese for all his support during this project. I have learned with him the features of a great scientist and hope to achieve one day his level of competence and knowledge. I would also like to thank Dr. Keiko Mizuno for all her help during my PhD. She has taught me a lot in molecular and behavioural techniques and her help was essential for the execution of every experiment in this thesis. I am also grateful to Dr. Diana Hanger for her role as a second supervisor, given important inputs in my project. I would like to thank as well Dr. Walter Lucchesi for the production of the viral vectors used in this project and for his teachings in life and music. Is also my pleasure and obligation to thank all the students that worked in this project before me, as for example Victoria Valls Comamala, for laying the ground foundations of my work. I am also grateful to Dr. Sachin Tiwari, Dr. Wajeeha Aziz, Anshua Ghosh and Vitor Paiva for creating a environment of friendship in our group, given me support with kind words and important suggestions.

I would like to thank all my friends from the JBC for making this PhD experience unique. Their friendship was many times a life jacket in a sea of problems.

A special thanks to my family for the unconditional support during my PhD. I owe all that I am to my family and this thesis is their achievement as much as mine.

And finally my eternal gratitude to the love of my life, Livia Freire Ferreira, who has sacrifice so much for this thesis. Her support, love and teachings in psychology have shapped this thesis as well as the man that I am.

Bibliographic production

Book Chapter

- Fabio A.B. Vigil and K. Peter Giese. **CaMKII autophosphorylation-dependent learning and memory.** Chapter of book: Novel Mechanisms of Memory. Springerbook-317288. Estimated Publication Date: November/2015.

Abstract in conference

- VIGIL, F. A. B. ; Lucchesi W. ; MIZUNO, K. ; COMAMALA, V. ; GIESE, K. P. . **The roles of endogenous CaMKII inhibitors in learning and memory**. In: BNA2013: FESTIVAL OF NEUROSCIENCE, 2013, London. BNA2013: FESTIVAL OF NEUROSCIENCE, 2013. v. 22. p. 263-263.
- FABIO, VIGIL ; KEIKO, MIZUNO ; WALTER, LUCCHESI ; VICTORIA, COMAMALA ; PETER, GIESE . **The roles of endogenous CaMKII inhibitors in learning and memory.** Frontiers in Systems Neuroscience, v. 8, p. 37, 2014.
- VIGIL, F.A.B. ; MIZUNO, K. ; Lucchesi W. ; COMAMALA, V. ; PETER, GIESE . **The roles of endogenous Calcium/ Calmodulin-dependent kinase 2 inhibitors in learning and memory.** In: Society for Neuroscience annual meeting 2014, Washington, DC, USA. 2014 Neuroscience Meeting Planner, 2014. v. 359.08. p. SS50-SS50.

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Abbreviations

Abbreviation	
A.U.	arbitrary units
AAV	adeno-associated virus
ABP	AMPA receptor-binding protein
AD	Alzheimer's disease
ADHD	attention-deficit/hyperactivity disorder
AIP	autocamtide-2-related inhibitory peptide
ALS	amyotrophic lateral sclerosis
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
AS	Angelman syndrome
ATRX	α-thalassemia X-linked mental retardation
Aβ	beta amyloid
BDNF	brain derived neurotrophic factor
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CaM	calcium/calmodulin complex
CaMKII	calcium/calmodulin-dependent kinase II
cDNA	complementary DNA
CFC	contextual fear conditioning
CNS	central nervous system
CR	conditional response
CREB	calcium/cAMP-response element binding protein
CS	conditioned stimulus
CT	threshold cycle
CYLD	cylindromatosis
DAPI	4',6-diamidino-2-phenylindole
DG	dentate gyrus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
DTT	dithiothreitol
ERK	extracellular-regulated kinase
FRET	fluorescence resonance energy transfer
FTLD	frontotemporal lobar degeneration
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC/mL	genome copies *per* milliliter
GFP	green fluorescent protein
Gtp	guanosine-5'-triphosphate
HPRT	hypoxanthine phosphoribosyltransferase
IKK	IκB kinase
IRES	internal ribosome entry site
ITR	inverted terminal repeats
L-LTP	late LTP
LTD	long-term depression
LTP	long-term potentiation
mRNA	messenger RNA

MWM	Morris water maze
NAc	nucleus accumbens
NF-κB	Nuclear factor-κB
NMDAR	N-methyl-D-aspartate receptor
osc/min	oscillations per minute
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PKA	protein kinase A
PRP	plasticity-related proteins
PSD	post-synaptic density
ras-GTP	ras-guanosine-5'-triphosphate
rAVV	recombinant adeno associated virus
RNA	ribonucleic acid
Rpm	revolutions per minute
RT-qPCR	real-time polymerase chain reaction
SDS	Sodium dodecyl sulfate
SERT	serotonin transporter
shRNA	short hairpin ribonucleic acid
SNK	Student-Newman-Keuls
SPS	single prolongued stress
STP	short-term potentiation
Tat	trans-acting activator of transcription
TBS	tris-buffered saline
TBST	tris-buffered saline and tween 20
TDP-43	TAR DNA binding protein-43
US	unconditioned stimulus

Chapter 1 Introduction

1.1 Learning and memory

Learning and memory are key abilities for the survival of our species and throughout the evolution of life on this planet one can follow the emergence of more complex learning/memory abilities corresponding to the phylogenetic hierarchy (Jensen, 1971). Consequently, these cognitive processes are not unique of our species and were adapted and diversified during the evolution of different species. Furthermore, it is plausible to assume that different species share learning/memory mechanisms. The cognitive process of learning and memory can be divided into three different processes: memory formation, memory storage and memory retrieval. Memory formation, or learning, as defined by Pavlov (1926) is the association of two distinct environmental stimuli resulting in similar behavioural response for both stimuli, nowadays called associative learning. However, the learning process is complex. For example, memory formation after one training trial and memory formation after repeated training trials have different biological substrates (Irvine et al., 2006). It is also known that different stimuli require different processing by the brain (Bentz and Schiller, 2015; McCarthy et al., 1997). Long-term memory formation requires a transient consolidation process shortly after training. Typically the cellular mechanism of consolidation is completed within 24 hours. Memory consolidation has first been proposed by Müller and Pilzecker (1900) based on their observation that, in humans, a newly learned information was disrupted by the learning of another information shortly after the original learning, but not if it happened a long period after learning of the first information. Müller and Pilzecker (1900) suggested that processes underlying new memories initially persist in a fragile state and consolidate over time. This hypothesis

was corroborated by the observation that blocking of previously learned information could be achieved by pharmacological inhibition of protein synthesis (Agranoff et al., 1966; Schafe and LeDoux, 2000). After consolidation memory is belived to be stored and maintained. Once information is stored it can be retrieved by an environmental stimulus which results in behavioural responses in accordance to the previous learned information. The retrieval of a memory could result in memory extinction or memory reconsolidation (Almeida-Correa and Amaral, 2014; Baldi and Bucherelli, 2015). The first evidence of memory reconsolidation process was presented by Misanin et al. (1968), by observation of an amnesic effect of electroconvulsive shock 24 hours after fear conditioning training dependent on the presentation of the conditioned stimulus. In other words, the associative memory between a neutral conditioned stimulus and an unconditioned stimulus (shock) was lost after electroconvulsive shock only if the memory was retrieved (Misanin et al., 1968). This observation challenged the long prevailing theory that memories once consolidated would no longer be labile. Recently, memory reconsolidation has been shown to be an important process for the maintenance and further strengthening of a memory (Fukushima et al., 2014; Lee, 2008). Memory extinction, on the other hand, is the learning of new environmental conditions that suppresses the previously learned conditioned response (Myers and Davis, 2007; Pape and Pare, 2010; Quirk and Mueller, 2008). Memory extinction was first described by Pavlov (1926), and it presents behavioural and molecular specificities that will be explained in more detail on subchapter “1.5.4 CaMKII and memory extinction”. Due to their opposite outcomes, memory reconsolidation and memory extinction are considered to be two opposing processes that have different biological substrates (Baldi and Bucherelli, 2015; Eisenhardt and Menzel, 2007; Suzuki et al., 2004). Nonetheless, there is also the possibility that memory reconsolidation and extinction

are actually two different outcomes of the same process and that they share biological substrates (Almeida-Correa and Amaral, 2014; Trent et al., 2015). Since most extinction protocols require different sessions of re-exposure to the context for extinction to occur, Fiorenza et al. (2011) even talks about reconsolidation of the extinction of a memory. The molecular bases of the different memory processes explained above will be discussed in detail further ahead in this thesis.

An important step in the understanding of learning/memory processes was the discovery of the electrophysiological phenomenon of long-term potentiation (LTP) (Bliss and Collingridge, 1993; Bliss and Lomo, 1973). LTP is a long-lasting increase in synaptic transmission that is induced by previous synaptic activity (Bliss and Collingridge, 1993; Bliss and Lomo, 1973). LTP is considered to be an important biological substrate for learning and memory (Gruart et al., 2015; Maren and Baudry, 1995; Teyler and Discenna, 1984). In terms of learning, it is known that fear conditioning training induces the occurrence of LTP (Rogan et al., 1997), as well as training in other tasks like trace eyeblink conditioning (Gruart et al., 2006) and inhibitory avoidance task (Whitlock et al., 2006). Additionally, genetic and pharmacological manipulations that impaired LTP induction also impaired memory formation, as observed by Giese et al. (1998) with a knock-in mutation in CaMKII and Gruart et al. (2006) with the blocking of the N-methyl-D-aspartate receptor (NMDAR). Regarding LTP and memory, Abraham et al. (2002) have shown that in the rat brain LTP can last for several months, which means that LTP is a feasible biological substrate for memory. Corroborating this hypothesis first Ling et al. (2002) have shown that protein kinase Mzeta (PKMz) is necessary for LTP maintenance. Later Pastalkova et al. (2006) showed that blocking of PKMz blocks LTP maintenance and also abolishes an established memory of a previously learned spatial information.

Indeed it is quite tempting to establish a comparison between LTP and the classical Hebbian postulation of a memory mechanism which states that “when neuron A repeatedly or persistently fires neuron B, then some metabolic or growth changes are induced so neuron A fires neuron B more efficiently” (Hebb, 1949). However, this comparison does not hold, since strictly speaking LTP is a synaptic event while Hebb’s postulate refers to neuronal firing.

Another biological substrate of memory is found in morphological changes at synapses. Synapses are the sites where two neurons establish a chemical communication and are composed of a presynaptic bouton in contact with a postsynaptic dendritic spine, more specifically with the postsynaptic density (PSD), which contains receptors, scaffold and signalling proteins (DeFelipe, 2006; Gu and Zheng, 2009). Dendritic spines are very plastic structures and their morphology can change due to synaptic activity (Yasumatsu et al., 2008). The function of these morphological changes are still poorly understood. Hung et al. (2008) observed that mice lacking Shank1, an important scaffold protein of the PSD, have more small dendritic spines and fewer larger dendritic spines, as well as, enhanced spatial learning but decreased memory maintenance. This seems to suggest that larger spines keep the memories while smaller spines enable learning. However, in a meta-analysis study by Marrone (2007) it was shown that a diversity of morphological changes in hippocampal dendritic spines is associated with different learning paradigms. Nevertheless, dendritic complexity and spine density were some of the parameters that were increased by different learning paradigms (Marrone, 2007). It seems clear as well that large spines have larger PSDs, and are more stable over time (Bourne and Harris, 2008). Finally, one of the most strong associations between synapses and cognition come from studies with

Alzheimer's disease patients were it was reported that the loss of synapses has the strongest correlation with the patient's cognitive decline (DeKosky and Scheff, 1990; Terry et al., 1991).

Regarding studies of biological substrates of learning/memory the hippocampus has been one of the most studied areas of the brain. The association between the hippocampus and learning/memory has been of great interest since the classical case of Henry Gustav Molaison, known widely as HM (Scoville and Milner, 1957). HM was a patient with memory disorder caused by a bilateral medial temporal lobectomy which surgically removed the anterior two thirds of his hippocampus, parahippocampal cortices, entorhinal cortices, piriform cortices, and amygdala in an attempt to cure his epilepsy (Scoville and Milner, 1957). Since then, various studies have shown that lesion or blocking of hippocampal activity has deleterious effects on learning and memory (Bannerman et al., 2004; Greene, 1971; Ricci et al., 2015; Smieskova and Svorad, 1965; Wang et al., 2015a; Yoon et al., 2008). As proposed by O'Keefe and Nadel (1978) the hippocampus seems to be the neural substrate of a cognitive map of space. In fact, various spatial memory tasks for rodents have been shown to be hippocampus dependent, for example the radial arm maze (Mitchell et al., 1982), T-maze (Rawlins and Olton, 1982), the Morris water maze (MWM) (Morris et al., 1982) and the contextual fear conditioning (CFC) (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). The prevailing theory was that memories were initially stored in the hippocampus but then 'transfered' to neocortex. This hypothesis was corroborated by impairment of contextual fear memory by hippocampal lesioning only if the lesion was done one day after training but not if it happened at a more distant time point (Anagnostaras et al., 1999; Kim and Fanselow, 1992). However, this theory has been put in check by the

observation by Goshen et al. (2011) that quick and selective blocking of the neuronal activity at the hippocampal sub-region CA1, by optogenetics, abolishes the recall of contextual fear memory even weeks after contextual fear training. Goshen et al. (2011) further observed that continuous blocking would trigger an adaptive shift of the memory to alternative structures of the brain, which could explain the independence of long-term contextual fear memories of the hippocampus previously reported by other authors (Anagnostaras et al., 1999; Kim and Fanselow, 1992).

CFC is a hippocampus-dependent Pavlovian conditioning task in which a neutral context [conditioned stimulus (CS)] is associated with an aversive unconditioned stimulus (US). After conditioning the CS evokes a learned fear response, the conditional response (CR) (Maren, 2008; Pavlov, 1926; Phillips and LeDoux, 1992). The CFC paradigm uses a mild foot-shock as US, freezing behaviour as CR and the trained context as CS (box size, texture, colour, smell…). Context in which the associative memory was formed has been proved to be an efficient stimulus to elicited memory recall as shown by presentation of the CR by the animal (Bouton, 1993; Bouton and Swartzentruber, 1986). Due to the fact that CFC memory can be formed with one trial only the CFC allows for the separated study of memory formation, consolidation and retrieval induced process. With respect to retrieval-induced process, CFC has been frequently used to study memory extinction by prolonged and repetitive exposition to the context without shock (Liu et al., 2015; Wang et al., 2015b). It can also be used to confirm extinction by reinstatement with a weak shock (Rescorla and Heth, 1975; Trent et al., 2015) or with a shock in a different context (Corcoran et al., 2013). One short re-exposure to the context without a shock is used as protocol to study memory reconsolidation (Lee, 2008; Lee et al., 2008). Besides being a useful tool for the study of learning and memory the CFC

paradigm has also been used as a tool for studying post-traumatic stress disorder (PTSD) (Eskandarian et al., 2013; Pinna and Rasmusson, 2014; Steiger et al., 2015) and generalized anxiety disorder (GAD) (Luyten et al., 2011). CFC memory is a hippocampus-dependent memory (Phillips and LeDoux, 1992). More specifically the dorsal hippocampus plays a key role in CFC memory. Lesion of the dorsal hippocampus causes retrograded amnesia of the context if done after CFC training, without affecting tone memory (Anagnostaras et al., 2001; Anagnostaras et al., 1999; Kim and Fanselow, 1992). On the other hand, the ventral hippocampus seems to have a role in contextual and cued fear memory (Anagnostaras et al., 2001; Bhardwaj et al., 2014; Zhu et al., 2014). The amygdala has also been shown to be relevant for contextual (Phillips and LeDoux, 1992) and cued fear memory (Nader et al., 2000; Phillips and LeDoux, 1992). Although studies with animal models like the CFC paradigm have helped establish the hippocampus, LTP and synaptic plasticity as biological substrates for fear learning and memory very little is known about the molecular substrates.

1.2 Calcium/Calmodulin-dependent kinase II

CaMKII is the major post-synaptic density (PSD) protein and accounts for 1-2% of total protein in the brain (Cheng et al., 2006; Erondu and Kennedy, 1985; Peng et al., 2004). CaMKII is a serine/threonine kinase composed of an auto-inhibitory regulatory domain, an N-terminal kinase domain and a C-terminal self-association domain (Chao et al., 2011; Hell, 2014). During resting state this enzyme is inactive due to blocking of the substrate binding site (S-site) and the catalytic domain, both found in the kinase domain, by the pseudosubstrate segment in the regulatory domain (Braun and Schulman, 1995a; Hell, 2014). The existence of two structurally different inactive states of CaMKII has been proposed by Stratton et al. (2013). His hypothesis is based on the differenct diameters of CaMKII when observed by electron microscopy (Kolodziej et al., 2000), small angle X-ray scattering (Rosenberg et al., 2005) or crystallography (Chao et al., 2011). The two states would exist in equilibrium with one state being more compact where the kinase domain is in high proximity with the self-association domain, whilst the other inactive state of the enzyme would be more open with the kinase and regulatory domains further apart from the self-association domain (Hell, 2014; Stratton et al., 2013). Only the second state would allow the binding of calcium/calmodulin complex (CaM) to the enzyme (Hell, 2014; Stratton et al., 2013). Binding with CaM causes a conformation change in CaMKII that unblocks the kinase domain from the inhibitory domain activating the enzyme (Colbran et al., 1989; Grant et al., 2008; Meyer et al., 1992). However, continuous activation of CaMKII by high levels of CaM might actually inhibit CaMKII and cause punctate deposition (Grant et al., 2008). A schematic representation of the two inactive states of CaMKII and activation by CaM can be seen in Figure 1.2-1. It is important to notice that CaMKII activation is regulated by calcium influx in the cell, but CaMKII activity also regulates calcium influx in the cell. The influx of calcium to a cell can

be facilitated by a 200-ms-long prepulse to +160 mV (Lee et al., 2006). This facilitation of calcium influx is abolished by pharmacological blocking of CaMKII kinase activity (Lee et al., 2006). Furthermore, Lee et al. (2006) observed that CaMKII coimmunoprecipitated with the calcium channel $Ca_v1.2$, and that $Ca_v1.2$ phosphorylation at serine 1512 and serine 1570 by CaMKII is necessary for voltage-induced facilitation of calcium influx. Binding of CaMKII to $Ca_v1.2$ has been shown to occur in the C and N terminal domains of this calcium channel, causing an increase of its expression at the cell surface (Hudmon et al., 2005; Simms et al., 2015). Other calcium channels have also been shown to bind to CaMKII causing an increase in calcium influx, like for example the calcium channel $Ca_v1.3$ (Jenkins et al., 2010) and $Ca_v2.1$ (Magupalli et al., 2013). CaMKII has a wide range of substrates and is involved in many aspects of cellular function, such as the regulation of ion channel function, neurotransmitter release, gene transcription, cytoskeleton organization and intracellular calcium homeostasis (Erondu and Kennedy, 1985; Hudmon and Schulman, 2002; Lisman et al., 2002; Lisman et al., 2012; Lucchesi et al., 2011; Tobimatsu and Fujisawa, 1989). In mammals 4 different isoforms of this enzyme are expressed: α, β, γ and δ isoforms (Gaertner et al., 2004; Tobimatsu and Fujisawa, 1989). The most abundant isoforms in the brain are α and β CaMKII (Bennett et al., 1983; Peng et al., 2004; Tobimatsu and Fujisawa, 1989). These two isoforms differ in their kinetic of interaction with CaM, with α isoform requiring approximately 3 times the concentration of CaM needed by the β isoform to achieve the same levels of activation (De Koninck and Schulman, 1998). βCaMKII, but not αCaMKII, is capable of interaction with F-actin (Fink et al., 2003; Okamoto et al., 2009). This unique ability of βCaMKII to interact with F-actin has been proposed to be important in the regulation of the size and shape of the dendritic spines (Fink et al., 2003) and CaMKII localization in the cell (Borgesius et al., 2011). βCaMKII and αCaMKII also have different expression patterns in

the brain. While the α isoform is the predominant isoform in hippocampus and neocortex, βCaMKII is most expressed in cerebellum (Cheng et al., 2006; Erondu and Kennedy, 1985; Peng et al., 2004). Furthermore, hippocampal levels of αCaMKII tend to increase with neuronal activity whilst βCaMKII levels tend to decrease (Thiagarajan et al., 2002). Comparison between βCaMKII knockout mice and αCaMKII knockout also seems to indicate different functions for these two isoforms. βCaMKII knockout mice have impaired LTP in hippocampal neurons, increased long-term depression (LTD) in cerebellar neurons, motor impairment, cognitive impairment, decreased anxiety-related behaviour and a developmental delay in body weight gain (Bachstetter et al., 2014; Borgesius et al., 2011; van Woerden et al., 2009). On the other hand αCaMKII knockout mice have impaired LTP and impaired spatial learning but no change in motor skills or body weight gain (Silva et al., 1992a; Silva et al., 1992b).

Nevertheless, those isoforms are usually associated with both isoforms creating a holoenzyme composed of 12 CaMKII subunits organized into two hexameric rings (Hoelz et al., 2003; Kolodziej et al., 2000; Rosenberg et al., 2005) (Figure 1.2-1). The 12 subunits mainly include α and β CaMKII, creating heteromers, but homomers consisting of only αCaMKII have been found (Bronstein et al., 1988b). In fact, rat forebrain CaMKII is composed of αCaMKII homomers and αCaMKII and βCaMKII heteromers with a ratio of 2 α subunits to 1 β (Brocke et al., 1999). The organization of this enzyme in a complex of subunits facilitates the occurrence of autophosphorylation. Among the known autophosphorylation sites of CaMKII are threonine 305 (T305) and threonine 306 (T306). Phosphorylation of these sites is believed to be inhibitory due to blocking of the CaM binding site, causing CaMKII to translocate out of the PSD area, and decreasing LTP and learning (Elgersma et al., 2002; Hanson and Schulman, 1992; Shen et al., 2000).

However, very little is known about these phosphorylation sites. The most studied autophosphorylation site of CaMKII is at threonine in position 286 (T286) present in αCaMKII. In βCaMKII this threonine is in position 287. Phosphorylation of the T286/287 sites seems to occur between neighbouring subunits of the same holoenzyme and requires binding of CaM to both subunits involved (Hanson et al., 1994; Mukherji and Soderling, 1994; Rich and Schulman, 1998) (Figure 1.2-1). On the contrary, T305/306 autophosphorylation seems to occur within the same subunit and independently of CaM (Mukherji and Soderling, 1994). Phosphorylation at T286/287 allows CaMKII to remain in an active state even in the absence of CaM, a CaM-independent state of activation (Hanson et al., 1994; Irvine et al., 2006; Miller et al., 1988). T286 autophosphorylation also enhances CaM binding to the enzyme with an increase of 1000 times in the affinity between these two molecules and an increase in release time from less than a second to hundreds of seconds (Meyer et al., 1992). This phenomenon has been called CaM trapping, and it might play an important role in the increase of CaMKII kinase activity after T286 phosphorylation (Hell, 2014; Irvine et al., 2006; Meyer et al., 1992). Indeed autophosphorylation of T286/287 allows for longer and stronger translation of calcium spike frequency into kinase activity (De Koninck and Schulman, 1998; Fujii et al., 2013). T286/287 autophosphorylation also changes CaMKII binding to other molecules. It, for example, decreases βCaMKII affinity for F-actin (Shen and Meyer, 1999) and increases holoenzyme binding to NMDAR (Bayer et al., 2001). These two binding changes due to T286/287 phosphorylation will be discussed in more detail further on this thesis.

Given the importance of the phosphorylation of T286/287 in CaMKII it is only logical to conclude that its dephosphorylation is also an important process for regulation of learning/memory. In fact, two different mathematical models have suggested that

dephosphorylation of CaMKII is a key process for controlling CaMKII activity and function in long-term changes at the synapses (Kubota and Bower, 2001; Zhabotinsky, 2000). The first phosphatases of CaMKII identified were the protein phosphatase PP1, PP2A, PP2B and PP2C, that were identified using immunoblotting, chromatography and phosphatase inhibitors/activators (Strack et al., 1997). Strack et al. (1997) also reported that most of CaMKII dephosphorylation was due to the activity of PP1 and PP2A, with PP1 activity more prominent in the PSD and PP2A in the cytosol. PP1 inhibition has been shown to be important for the induction of CaMKII-dependent LTP, as shown by exogenous and endogenous inhibition of PP1 (Blitzer et al., 1998; Brown et al., 2000). Furthermore, PP1 hippocampal mRNA levels were increased in animals with poor learning performance in the MWM task (Haege et al., 2010). Hippocampal PP1 and PP2A are also necessary for induction of LTD and associated dephosphorylation of calcium/cAMP-response element binding protein (CREB) (Mauna et al., 2011). In spite of that, the function of PP1 and PP2A in learning and memory cannot be conclusively associated with its dephosphorylation of CaMKII, since PP1 and PP2A have also been shown to regulate posttranslational modifications of histones with effects on learning and memory (Koshibu et al., 2011; Preethi et al., 2014). Another CaMKII phosphatase was described by Ishida et al. (1998a) and named CaMKIIPase. Initially thought to be a phosphatase specific for CaMKII, CaMKIIPase was later found to also dephosphorylate CaMKI and CaMKIV (Ishida et al., 1998b). This serine/threonine phosphatase has its activity regulated by Mn^{2+} levels and its substrate specificity seems to be determined by the substrate's tertiary and secondary structure, more than by its primary structure (Ishida et al., 1998a; Ishida et al., 2001). To my knowledge, until the present date no direct evidence of CaMKIIPase having a function on learning and memory has been reported.

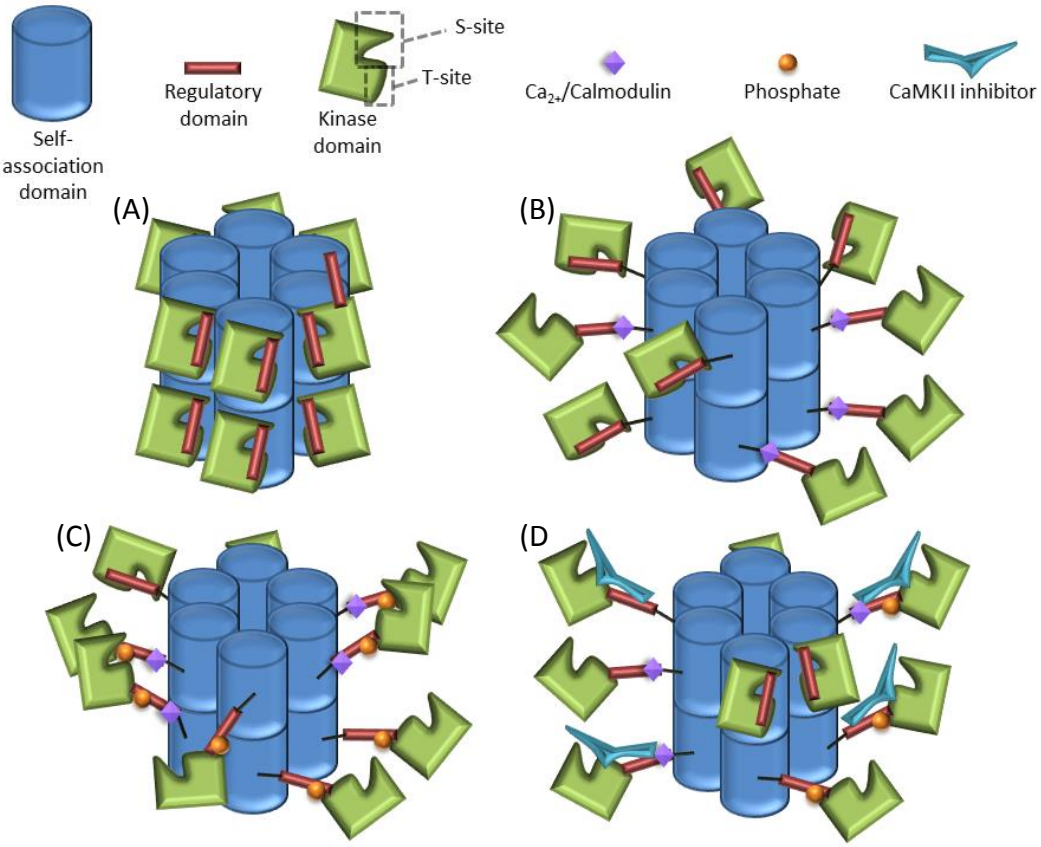

Figure 1.2-1 CaMKII holoenzyme

The figure shows a schematic representation of CaMKII holoenzyme in different states of activation. On the top of the figure the different domains of CaMKII subunits and interacting molecules are shown. (A) This panel shows CaMKII in its most inactive state, with the regulatory domain connected to the T-site (a hydrophobic region in the kinase domain) and S-site of the kinase domain. Both domains are packed in close proximity to the self-association domain (Braun and Schulman, 1995a; Hell, 2014). (B) This panel shows the CaMKII in two different states of activation. One is an inactive state were the regulatory domain is still blocking the kinase domain, however these domains are found further apart from the self-association domain. Only this more open state of inactivation allows CaM to bind to CaMKII (Hell, 2014; Stratton et al., 2013). Binding of CaM to CaMKII releases the kinase domain from the regulatory domain exposing the S-site, as represented in this panel (Colbran et al., 1989; Grant et al., 2008; Meyer et al., 1992). (C) Once activated by CaM, CaMKII will start the autophosphorylation of its subunits at the T286/T287 site. CaM bound to CaMKII is necessary for this phosphorylation to occur but not for the maintenance of it. This means that CaMKII remains active and phosphorylated at T286/T287 even after CaM is no longer bound to it (Hanson et al., 1994; Irvine et al., 2006; Miller et al., 1988). (D) This panel represents the inhibition of CaMKII by the endogenous inhibitors CaMK2N1 and CaMK2N2. The inhibitors of CaMKII bind to the enzyme at the regulatory domain and the T-site, blocking access of the substrate to the S-site but without directly blocking the T286/T287 site (Chang et al., 2001; Vest et al., 2007).

1.3 The T286 autophosphorylation theory and LTP

Due to the ability to switch from a CaM-dependent to a CaM-independent state of activation (bistability), CaMKII has been suggested to act as a memory molecule preserving “memories” of strong calcium signals (Lisman, 1994). Before the publication of his paper in 1994 (Lisman, 1994), Lisman used mathematical models to study if CaMKII bistability was a good candidate to store long-term memory (Lisman and Goldring, 1988a; Lisman and Goldring, 1988b). His models suggested that CaMKII autonomous activity could be stable enough to maintain LTP and memories for a lifetime. These models were based in the hypothesis that T286 autophosphorylation of neighbouring subunits could overcome the dephosphorylation rate of CaMKII, maintaining the enzyme activated for a long time. Furthermore, it would also be necessary that degraded subunits were replaced by newly synthesized subunits that would subsequently be phosphorylated at the T286/287 site by their neighbouring subunits (Irvine et al., 2006; Lisman, 1994; Lisman and Goldring, 1988a; Lisman, 1985; Lisman and Goldring, 1988b). However, a more recent computational model proposes that this might not be the case. Using analysis of the stochastic transition between the ON and OFF states of CaMKII as a consequence of T286 phosphorylation, Miller et al. (2005) have reported the autophosphorylation to be stable only for a few years.

However, Miller et al. (2005), Lisman and Goldring (1988a) and Lisman and Goldring (1988b) models are unlikely to be corrected. Various authors have suggested that LTP induction only results in a transient increase in CaMKII autonomous activity, lasting for only a few minutes (Fujii et al., 2013; Lee et al., 2009; Lengyel et al., 2004). For example, at the level of single synapses, Fujii et al. (2013) and Lee et al. (2009) used two-photon glutamate uncaging pulses to induce LTP and fluorescence resonance

energy transfer (FRET)-based sensor to study CaMKII activity and observed that LTP induction leads only to transient increase of CaM-independent activity. Lengyel et al. (2004) analysed CaMKII CaM-independent kinase activity by quantifying incorporation of radioactive phosphorus in CaMKII's substrate autocamide-2 in the presence of EGTA after LTP-inducing electric stimulation and did not detect any changes in autonomous CaMKII kinase activity. According to the authors, this might be due to the fact that a change in autonomous CaMKII activity was too transient to be detectable, too small to be measured or it may not occur. Therefore, the same authors used a chemical stimulation to induce LTP and short-term potentiation (STP) and they noticed that both protocols produce very similar effects on CaMKII autonomous activity. These stimulations led only to a transient increase in CaMKII autonomous activity that lasts up to 5 minutes. Interestingly, T286 autophosphorylation, measured by immunoblotting, remained elevated for longer. STP would be accompanied by an increase in T286 autophosphorylation that disappeared after 10 min. LTP induction, on the other hand, resulted in increased T286 autophosphorylation even after 60 min (Lengyel et al., 2004). Lengyel et al. (2004) observations not only confirm that LTP induction results in transient increase in CaMKII kinase activity as it also reveals that CaMKII autonomous kinase activity and T286 autophosphorylation are not synonyms. Indeed, Tzortzopoulos and Torok (2004) accessed the induction of CaMKII kinase activaty by T286 phosphorylation, using smooth muscle myosin light chain as substrate and fluorometric quantyffication of kinase activaty, and observed that the majority of kinase activaty induced by T286 phosphorylation was actually dependent of CaM. Thus, the contradiction between the empirical observations of LTP-induced CaMKII kinase activaty (Fujii et al., 2013; Lee et al., 2009; Lengyel et al., 2004) and Miller et al. (2005), Lisman and Goldring (1988a) and Lisman and Goldring (1988b) models might be due to the fact that these models fail to

consider the dependence of T286 induction of CaMKII kinase activaty to CaM availability (Tzortzopoulos and Torok, 2004). These models also do not consider how other post-translational changes also regulate CaMKII kinase activaty. For example, A different mechanism of CaMKII CaM-independent activity has been described by Coultrap and Bayer (2014). αCaMKII seems to be regulated by nitrosylation of cysteines in the positions 280 and 289. These nitric oxide-induced nitrosylations at C280 and C289 generate an autonomous state of CaMKII activity, which is independent of T286 autophosphorylation and actually reduces T286 autophosphorylation. Interestingly, this mechanism has no impact on T305 autophosphorylation (Coultrap and Bayer, 2014).

Although Miller et al. (2005), Lisman and Goldring (1988a) and Lisman and Goldring (1988b) models are unlikely to be correct they inspired many studies on the role of CaMKII autophosphorylation including the generation of knock-in mutant mice that contained a single point mutation in position 286, inhibiting the autophosphorylation by replacing threonine by an alanine (T286A) (Giese et al., 1998). The study of T286A mutant mice revealed that this autophosphorylation of αCaMKII is required for the induction of NMDAR-dependent LTP at excitatory hippocampal CA1 synapses (Giese et al., 1998). Interestingly, in the first two weeks of postnatal life αCaMKII autophosphorylation is not required for CA1 LTP. There is a developmental switch changing CA1 LTP from protein kinase A (PKA)-dependence to CaMKII-dependence (Yasuda et al., 2003). Further, LTP in dentate gyrus (DG), a brain area where constantly new neurons are born, does not require αCaMKII autophosphorylation. Wu et al. (2006) observed that the pharmacological block of CaMKII activity does not impair DG LTP. It is necessary to block PKA or MAPK/ERK kinase (MEK) together with CAMKII to achieve impairment in LTP. This suggested an additional mechanism of LTP regulation

present in these cells. The same results were observed in T286A DG cells. The mutation alone is not capable of blocking LTP. LTP is impaired only when PKA or MEK are also blocked in these cells (Cooke et al., 2006). In accordance with this observation, in obese Zucker rats, an obesity model, and also in a rat model of hypothyroidism αCaMKII T286 autophosphorylation is reduced and CA1 LTP is impaired. On the other hand, the same models do not have impairred DG LTP (Alzoubi et al., 2005; Gerges et al., 2005).

It is important to consider that even in a specific area of the hippocampus, like CA1, different types of synapses have different dependency of αCaMKII T286 autophosphorylation for LTP formation. The results presented earlier which show a dependency of T286 αCaMKII autophosphorylation for LTP occurrence in CA1 area were conducted at excitatory synapses in pyramidal cells (Giese et al., 1998), but NMDAR-dependent LTP at inhibitory synapses can be induce in the absence of T286 autophosphorylation (Lamsa et al., 2007). This NMDAR-dependent LTP in inhibitory neurons is probably dependent of non-α isoforms of CaMKII, since αCaMKII is expressed only in glutamatergic neurons (Liu and Jones, 1996). However, since Lamsa et al. (2007) reported in the same article that the induction of this NMDAR-dependent LTP in inhibitory neurons can be blocked by unspecific pharmacological inhibition of calcium/calmodulin-dependent kinases, the relevance of other CaMKII isoforms cannot be discarded. Regarding neocortical cells, *in vitro* and *in vivo* experiments, using T286A animals, demonstrated a dependency on CaMKII T286 autophosphorylation for LTP induction (Hardingham et al., 2003).

Taken together, functional studies have established that the T286 autophosphorylation has a fundamental role for the induction of some forms of

NMDAR-dependent LTP, and it is unlikely that autonomous CaMKII activity maintains LTP, as this activity lasts only for a very short duration.

1.4 CaMKII and learning

Considering that CaMKII plays a pivotal role on hippocampal LTP induction, as mentioned above, one should expect that this enzyme would also be necessary for learning of a hippocampus-dependent task. Indeed the T286A mutant mice have one of the most severe learning deficits ever described for mice in the MWM task (Giese et al., 1998; Need and Giese, 2003). Loss of T286 autophosphorylation seems to block strategy and spatial learning (Need and Giese, 2003). Environmental enrichment and handling do not overcome the spatial learning deficits in the T286A mutants (Need and Giese, 2003). Place cell recordings in T286A mutants have shown that the autophosphorylation is required for place cell spatial selectivity, stability and fine tuning (Cacucci et al., 2007; Cho et al., 1998). The T286A mutant mice are also impaired in memory formation in passive avoidance task, as well as cued and contextual fear conditioning (Irvine et al., 2005). Gokcek-Sarac et al. (2013) reported that animals with "good" learning performance in the 12-arm radial maze have higher levels of T286-autophosphorylated αCaMKII in comparison with animals with "poor" performance and this difference is not simply the result of altered αCaMKII total levels. Lisman et al. (2012) proposed a model for the role of CaMKII in LTP induction based on the modulation of glutamate receptor activity. In short, CaM is produced by calcium influx through NMDAR. CaM activates CaMKII holoenzymes that are present in the synapse, therefore, initiating T286 and T287 autophosphorylation. The T287 phosphorylation of βCaMKII subunits and CaM binding releases the holoenzyme from binding to F-actin, allowing CaMKII to freely diffuse in the

synapse. Once CaMKII reaches the PSD area, its localization is stabilized by binding to the NMDAR. CaMKII can actually bind to two different sites of the NMDAR. One site depends on CaMKII's association to CaM (within residues 1,120-1,480 of the NMDAR) and a second binding site depends on T286 phosphorylation (residues 839-1,120) (Bayer et al., 2001). Bayer et al. (2001) even showed that, besides being anchored in the PSD area by the interaction with the NMDAR, this interaction might actually create an autonomous activity state of CaMKII, independent of CaM and also independent of autophosphorylation of its subunits. Although this is a very interesting finding by Bayer et al. (2001), the data were obtained from *in vitro* experiments and such mode of activation still has not been shown to occur *in vivo*. Autophosphorylated CaMKII in the PSD seems to increase glutamatergic transmission by phosphorylation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) subunit GluA1 at serine 831. This phosphorylation increases AMPA receptor conductance (Derkach et al., 1999). Nevertheless, under basal or stimulated conditions only a minor percentage of all the GluA1 subunits in the synapses seems to be phosphorylated at serine 831 (Hosokawa et al., 2015). CaMKII can also regulate the number of AMPAR in the synapses by phosphorylation of the protein stargazin. Stargazin is an AMPAR binding protein and once it is phosphorylated it also binds to the scaffold protein PSD-95, enhancing the number of AMPAR in the PSD area (Opazo et al., 2010; Tomita et al., 2005). This way an initial burst of calcium into the cell would lead to an increase in glutamatergic transmission. Such mechanism could be important for the induction of LTP. A schematic representation of this process can be seen in Figure 1.5-1. However, if we consider the turnover and dephosphorylation of proteins this mechanism is most likely transient and it cannot account for LTP maintenance, in accordance with the LTP studies already mentioned. Another phosphorylation site of GluA1 that has been identified as substrate

for CaMKII kinase activity is the serine at position 567 (Lu et al., 2010). The phosphorylation of this site by CaMKII has been shown to regulate AMPAR trafficking to the synapse (Lu et al., 2010). GluA1 S567 phosphorylation is induced by chemical or electrical induction of LTD and results in a decrease of AMPAR in the synapse (Coultrap et al., 2014; Lu et al., 2010).

CaMKII interacts not only with the glutamatergic system, as presented above, but it also regulates and/or is regulated by other neurotransmitters like serotonin, glycine and gamma-aminobutyric acid (GABA). In terms of serotonin, it is known that inactivation of CaMKII by KN-93 increases serotonin signalling by decreasing the interaction of plasma membrane serotonin transporter (SERT) with the protein syntaxin 1A (Ciccone et al., 2008). Colocalization of SERT and αCaMKII has been observed by Steinkellner et al. (2015), who also observed that inhibition of αCaMKII impaired amphetamine-induced SERT-mediated substrate efflux. Additionally, Asaoka et al. (2015) reported that treatment of organotypic raphe slice cultures with the inhibitor of histone deacetylases trichostatin A caused an increase in serotonin release and an increase in αCaMKII mRNA levels. Inhibition of CaMKII by KN-93 and KN-62 prevented the trichostatin-induced increase in serotonin release (Asaoka et al., 2015). It has also been shown that pharmacological activation of the serotonin receptor 5-HT_{1A} impairs learning of the passive avoidance task and reduces the learning-induced increase in hippocampal CaMKII levels, CaMKII T286 phosphorylation, and CaMKII calcium-independent kinase activity (Moyano et al., 2004). Furthermore, inhibition of 5-HT_{1A}, by treatment with the antagonist WAY-100635, reversed the effects of 5-HT_{1A} activation in learning and in hippocampal CaMKII total levels, phosphorylation and kinase activity

(Moyano et al., 2004). Taken together these observation suggest that CaMKII regulates the serotonergic system as it is regulated by it.

Regarding GABA and glycine, CaMKII inhibition with KN-93, after unilateral cochlear ablation, facilitates GABA release in the medial nucleus of the trapezoid body and glycine release in the dorsal cochlear nucleus (Zhang et al., 2004). However, KN-93 treatment after unilateral cochlear ablation decreased glycine release in the ipsilateral medial superior olive and in the lateral superior olive, suggesting different function of CaMKII on glycine regulation within different regions of the brain (Zhang et al., 2004). Moreover, activation of CaMKII in synaptosomal membrane fractions, by treatment with calcium and calmodulin, increased muscimol binding to $GABA_A$ receptor, and CaMKII inhibition with KN-93 reversed this effect (Churn and DeLorenzo, 1998). Blocking of CaMKII activity with KN-93 or tatCN21 also blocked learning induced $GABA_A$-mediated miniature inhibitory postsynaptic potential in cortical neurons, which is suggested to be important for preventing uncontrolled synaptic strengthening and cortical overactivation (Ghosh et al., 2015). CaMKII seems to increase $GABA_A$ insertion in the membrane by phosphorylation of serine 383 within the β3 subunit of the $GABA_A$ receptor (Saliba et al., 2012). Marsden et al. (2010) has suggested that αCaMKII translocates to inhibitory synapses and regulates $GABA_A$ levels in the membrane after moderate NMDAR activation, while a stronger activation of NMDAR would favour CaMKII migration to excitatory synapses.

It is important, however, to consider that the inhibitors of CaMKII used in most of the experiments cited above, KN-93 and KN-62, have been shown to not be specific inhibitors of CaMKII affecting other molecules as well (Enslen and Soderling, 1994; Mochizuki et al., 1993; Redondo et al., 2010; Wayman et al., 2008).

Another possible molecular explanation for a role of CaMKII in learning emerges from the synaptic tag hypothesis (Figure 1.5-1). The synaptic tag theory proposes that during early LTP, a short lasting form of LTP, synapses can be "tagged". Upon future stimulation of the neuron plasticity-related proteins (PRP) can be synthesized in the soma, which then freely diffuse into the dendrites but will only be taken up by previously tagged synapses to induce long lasting LTP (Frey and Morris, 1997). Redondo et al. (2010) have shown that although CaMKII is not necessary for production of PRP it is necessary for the tagging of the synapses. Although very interesting, Redondo et al. (2010)'s observation has a limitation. KN-93, a pharmacological blocker, was used to block CaMKII function. As shown in their article, this inhibitor is not specific for CaMKII, so the authors analysed different doses of KN-93 and decided to use a concentration of 1µM. At this concentration KN-93 has a high affinity for CaMKII but there is still some inhibition of CREB phosphorylation. So the effects observed in synapse tagging by KN-93 treatment cannot be considered to be dependent of CaMKII activity only. For a more complete discussion on the matter of the lack of specificity of KN-93 see Wayman et al. (2008).

Another possible learning-related mechanism for CaMKII is metaplasticity. The state of activation of a neuron is defined by a balance between excitation and inhibition, between LTP and LTD. This balance is taken in account in the metaplasticity theory. Metaplasticity, which is commonly defined as "plasticity of synaptic plasticity", involves the alteration of the threshold for LTP and/or LTD induction in response to the previous history of synaptic activity from a specific synapse (Hulme et al., 2013). In other words, metaplasticity is a shift in the balance between LTP and LTD due to an activity trigger (priming). Such mechanism can be important to prevent the saturation of LTP in

stimulated synapses as to filter off subthreshold priming activities at synapses (Abraham and Tate, 1997). This learning mechanism has been proposed to be CaMKII dependent by Mayford et al. (1995). Those authors have suggested a role for CaMKII autonomous activity in metaplasticity by creating a different mutation on the T286 position of αCaMKII. Mayford et al. (1995) expressed a transgene that had the threonine in this site changed to an aspartic acid (T286D) mimicking the autophosphorylation. The transgene was expressed in addition to the endogenous CaMKII gene. As a result a metaplasticity phenotype was caused, favouring LTD over LTP. However, a later study conducted by Bejar et al. (2002) with the T286D mutant animals revealed that these mutants have altered expression of many proteins, including the inhibitory neuropeptide Y. Because of this, the metaplasticity phenotype in these mutants cannot be assigned directly to altered autonomous activity of CaMKII. Additionally Li et al. (2014) have observed that for a priming effect to occur a shift from a CaMKII dependent mechanism to a protein kinase Mzeta dependent mechanism may have to occur. Although it is still too early to fully understand the role of CaMKII in metaplasticity phenomenon, the study by Mayford et al. (1995) was a very important first step. More recently, Coultrap et al. (2014) have shown that αCaMKII is important for both LTP and LTD. In their experiment it was observed that T286 phosphorylation was required for the induction of LTP and LTD. While in LTP CaMKII appears to phosphorylate the AMPAR subunit GluA1 at S831 increasing its conductance, after LTD induction CaMKII phosphorylates GluA1 at S567, a phosphorylation site known to reduce synaptic GluA1 localization (Coultrap et al., 2014). Considering CaMKII as a metaplasticity switch, its control of the balance between LTP and LTD should respond to the history of stimulation of a neuron. Indeed continuous stimulation of CA1 cells from T286A mutant mice showed a decrease in the slow component of post-burst after-hyperpolarisation (Sametsky et al., 2009). As a

consequence of continued stimulation wild-type cells have an increased after-hyperpolarisation, which is a negative feedback for neuronal firing. Since T286A neurons have a reduced after-hyperpolarisation response, this suggests that T286 autophosphorylation might be a mechanism for downregulating the excitability of neurons as a response to a history of repetitive stimulation (Sametsky et al., 2009). Regarding weak stimulation, T286A cortical neurons have a higher threshold for intensity of the stimulus required to elicit a post-synaptic response (Pattinson et al., 2006) corroborating with the hypothesis of a role for CaMKII in metaplasticity. In fact, T286 autophosphorylation is widely accepted as important biological substrate for learning, so that it is used as a tool to study the neuroanatomical substrates of learning. For example, Cox et al. (2014) have used the T286 phosphorylation to assess how unrestricted and restricted learning of a new environment leads to different patterns of activation in the hippocampus. However, further studies with the T286A mice have shown that there might be a non-CaMKII dependent learning mechanism.

T286A animals show impaired learning but prolonged training can overcome this impairment. The mutants are capable of forming memories after contextual and cued fear conditioning once they were exposed to prolonged training protocols (Irvine et al., 2011; Irvine et al., 2005). Furthermore Ohno et al. (2005) observed that T286A animals were able to learn trace eyeblink conditioning normally. It is worth noting that trace eyeblink conditioning requires a long training protocol. This T286A-independent memory formation mechanism seems to be dependent on the generation of multi-innervated dendritic spines, which are postsynaptic spines that communicate with more than one presynaptic spine (Radwanska et al., 2011). It was also observed that long lasting LTP can be induced in CA1 pyramidal cells of T286A animals by repeated 100Hz

stimulation but this LTP was not synapse specific (Villers et al., 2014). The same authors also described that synaptse specific LTP can be achieved if the neurons are previously incubated for 2 hours with rapamycin, an inhibitor of mTOR-dependent translation, suggesting a possible change in the mechanism of LTP induction to a T286-independent mechanism (Villers et al., 2014).

1.5 CaMKII and memory

Although the relevance of CaMKII in learning has been established and widely accepted, its role in memory maintenance is still a matter of discussion. Buard et al. (2010) has shown that blocking CaMKII activity with systemic injection of the CaMKII inhibitor tatCN21, 1 hour before contextual fear conditioning testing session, had no effect on memory storage. The transient effect of LTP induction on CaMKII autonomous activity (Fujii et al., 2013; Lee et al., 2009; Lengyel et al., 2004), already mentioned, also seems to indicate an independence of memory maintenance to CaMKII activity. Despite these indications CaMKII has been proposed to be important for memory maintenance. The inhibition of CaMKII kinase activity by low doses of the synthetic peptide, CN21, had no effect on LTP maintenance. However, higher doses resulted in blocking CaMKII binding to the NMDAR and this impaired LTP maintenance, suggesting a more structural role for CaMKII (Sanhueza et al., 2011).

1.5.1 Structural roles of CaMKII

Sanhueza and Lisman (2013) put forward a model for the molecular basis of CaMKII in memory that combines the kinase activity of CaMKII and a structural role of

the enzymatic complex. Once again it all starts with calcium influx through NMDAR, which creates CaM that activates CaMKII. Activated CaMKII passes through autophosphorylation at T286 and T287 and migrates to the PSD where it binds to NMDAR. This first part accounts for the need of kinase activity. When bound to the NMDAR in the PSD the CaMKII complex would work like a scaffolding protein, interacting with the NMDAR and actinin in the PSD area. This complex would then bind to densin, delta-catenin, AMPAR-binding protein (ABP) and N-cadherin in an even larger complex. The ABP protein could anchor AMPAR in the PSD area, while N-cadherin creates a physical trans-synaptic bound that enlarges synapses both pre and postsynaptically (Sanhueza and Lisman, 2013). A schematic representation of this mechanism is presented on Figure 1.5-1.

CaMKII has also been proposed to be important in dendritic spine plasticity, which is considered a relevant mechanism for memory maintenance (Hell, 2014; Jourdain et al., 2003; McVicker et al., 2015; Okamoto et al., 2009; Okamoto et al., 2007). βCaMKII, but not αCaMKII, is capable of binding to and crosslinking F-actin filaments increasing their stability (Fink et al., 2003; Lin and Redmond, 2008; Okamoto et al., 2009; Okamoto et al., 2007). Upon CaM-dependent activation of βCaMKII and subsequent T287 autophosphorylation βCaMKII is displaced from F-actin causing the enzymatic complex to migrate to the PSD area (Lin and Redmond, 2008; Shen and Meyer, 1999). Furthermore this dissociation allows actin polymerization, increasing spine size and numbers (Fink et al., 2003; Jourdain et al., 2003; Okamoto et al., 2007). Because of this Okamoto et al. (2009) have suggested a model in which in a basal state of activation F-actin is bundled by βCaMKII. This interaction maintains the stability of spine structure. Upon stimulation releasing of βCaMKII from F-actin allows the polymerization of the

cytoskeleton leading to spine enlargement. Afterwards the T287 site is dephosphorylated and CaMKII complex binds again to F-actin and stabilizes the spine in this enlarged size. This could be an important mechanism for memory formation and/or maintenance by regulating the morphology of dendritic spines (Okamoto et al., 2009). A schematic representation of this process can be seen in Figure 1.5-1.

Structural role of CaMKII in the synapse morphology might also include αCaMKII by interaction with α-actinin and densin, other cytoskeleton molecules. Both αCaMKII and βCaMKII bind to α-actinin by a segment in the CaMKII autoinhibitory domain, the same that binds to CaM (Hell, 2014; Robison et al., 2005b; Walikonis et al., 2001). Indeed, CaM and α-actinin compete for binding to CaMKII (Robison et al., 2005a). CaMKII binding with α-actinin increases binding of this enzyme with F-actin (Jalan-Sakrikar et al., 2012) and is likely to play a role in CaMKII localization at the synapse (Hell, 2014). T286 phosphorylation does not affect CaMKII binding with α-actinin but phosphorylation of CaMKII at the T306 or T305 sites does (Jalan-Sakrikar et al., 2012; Robison et al., 2005a). While phosphorylation of T306 directly blocks binding with α-actinin, T305 phosphorylation might have an indirect effect, protecting CaMKII binding with α-actinin (Hell, 2014; Jalan-Sakrikar et al., 2012). Concerning CaMKII binding to densin, densin binds to the kinase domain of αCaMKII and βCaMKII (Jiao et al., 2011) and to the C-terminal of αCaMKII but not βCaMKII (Strack et al., 2000). Binding of densin to the αCaMKII C-terminal is independent of CaMKII activation, but binding of densin to the kinase domain is dependent on either CaM or T286 autophosphorylation (Jiao et al., 2011). Although the function of densin/CaMKII binding is not well understood a larger structure including CaMKII, F-actin, α-actinin and densin has been proposed by Hell (2014). Such configuration might help explain why densin knockout mice do not have a

decrease in CaMKII levels at spines or PSDs under basal conditions (Carlisle et al., 2011), since F-actin and/or α-actinin might still be sufficient to regulate properly CaMKII's localization (Hell, 2014).

1.5.2 CaMKII and memory consolidation

Learned information needs to undergo a consolidation process to form a long-term memory. This consolidation process requires gene transcription and protein synthesis (Bourtchouladze et al., 1998; Jarome and Helmstetter, 2014; Parsons et al., 2006; Schafe and LeDoux, 2000). In addition, not only one but at least two separate events of protein synthesis are required for memory consolidation (Bourtchouladze et al., 1998). It is known that the consolidation process requires the expression of brain derived neurotrophic factor (BDNF) (Lee et al., 2004) and also the transcription factor CREB (Bernabeu et al., 1997; Souza et al., 2012; Stevens, 1994). BDNF and CREB have been suggested to regulate each other by a feedback loop in a process that seems to be necessary for memory consolidation (Bambah-Mukku et al., 2014). CaMKII might play a role in this consolidation process by regulation of CREB phosphorylation (Souza et al., 2012). Corroborating with this hypothesis Chen et al. (2012) have observed a memory consolidation dependence to CaMKII-BDNF-CREB pathway. By pharmacologically blocking the glucocorticoid receptor in the hippocampus Chen et al. (2012) observed an amnesic effect on the passive avoidance task. The same animals also presented a reduction on CREB and αCaMKII phosphorylation. Additionally, intra-hippocampal injection of BDNF rescued the impairment in memory maintenance and αCaMKII and CREB phosphorylation (Chen et al., 2012). How the two cytoplasmic enzymes αCaMKII and βCaMKII could regulate the nuclear factor CREB has remained a mystery for a long time, until recent publication by Ma et al. (2014a) showing that βCaMKII phosphorylates

γCaMKII, which would transport CaM to the nucleus. Once in the nucleus γCaMKII releases CaM causing the activation CaMKK and subsequent activation of CaMKIV. Finally CaMKIV can directly phosphorylate CREB (Ma et al., 2014a; Ma et al., 2015). CaMKII regulation of CREB activity seem to be directly regulated by calcium influx trough Ca_v1 family of calcium channels (Wheeler et al., 2012).

Another known biological substrate of memory consolidation is the immediate early gene c-fos. C-fos mRNA levels were observed to be up-regulated, in the hippocampus, 1 hour after CFC training (Huff et al., 2006). Exploration of a new context also increases c-fos levels in the hippocampus after 1 hour (Radulovic et al., 1998). This CFC training-induced expression of c-fos seems to be regulated by the extracellular-regulated kinase (ERK), as observed by concomitant changes in c-fos levels and ERK phosphorylation after pharmacological manipulation of NMDAR activation (Gao et al., 2010). CaMKII interacts with the synaptic GTPase-activating protein (SynGAP). Through a cascade of signals SynGAP's interaction with CaMKII inhibits protein synthesis, with SynGAP preventing the ras-guanosine-5'-triphosphate (ras-GTP) formation that is necessary for ERK activation (Wang et al., 2013). Additionally, this signalling pathway is critical for synaptic plasticity, as shown by SynGAP blocking (Wang et al., 2013). Besides activating SynGAP, CaMKII also regulates SynGAP distribution to the PSD area (Yang et al., 2013). CaMKII might indirectly regulate c-fos expression throughout SynGAP's regulation of ERK activation.

Not only protein synthesis but also protein degradation seems to be important for memory consolidation (Artinian et al., 2008; Jarome and Helmstetter, 2014; Jarome et al., 2011; Lopez-Salon et al., 2001). CaMKII may also play a role in consolidation driven protein degradation. T286 autophosphorylation increases αCaMKII's affinity to

proteasome and promotes proteasome recruitment to the PSD area (Bingol et al., 2010). CaMKII can also phosphorylate proteasome subunit Rpt6 on serine 120 and increase its activity (Djakovic et al., 2009; Jarome et al., 2013). Moreover the phosphorylation of Rpt6 seems to decrease synaptic strength by impairing miniature excitatory postsynaptic current (Djakovic et al., 2012). CaMKII also phosphorylates the protein cylindromatosis (CYLD). Once phosphorylated, CYLD is activated and facilitates proteasomal degradation of proteins by removing K63-linked polyubiquitins from target proteins (Thein et al., 2014). A schematic view of this process is shown on Figure 1.5-1.

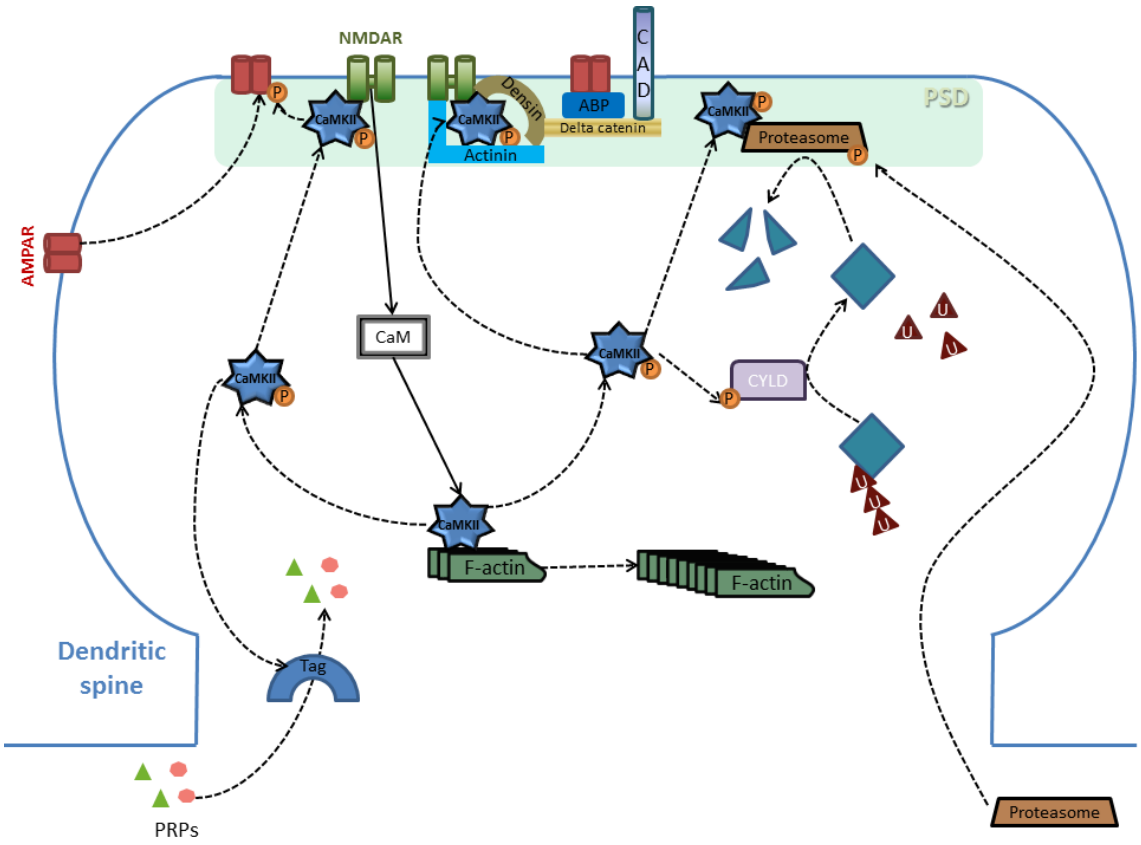

Figure 1.5-1 Schematic representation of the molecular mechanisms of CaMKII's function in the dendritic spine
A schematic representation of possible molecular bases for the effects of CaMKII heteromer complex on the dendritic spine function. The figure shows that after activation of the holoenzyme by CaM, this enzymatic complex will start to autophosphorylate and dissociate from F-actin (Lin and Redmond, 2008; Shen and Meyer, 1999). This allows the diffusion of CaMKII into the PSD as well as F-actin remodelling (Borgesius et al., 2011; Fink et al., 2003). Once phosphorylated at position T286 αCaMKII migrates to the PSD, binds to NMDAR (Bayer et al., 2001) and phosphorylates the AMPAR (Derkach et al., 1999). The phosphorylation of the AMPAR will increase its conductance and stabilize its localization in the PSD area (Derkach et al., 1999; Opazo et al., 2010; Tomita et al., 2005). CaMKII could also form a bigger complex involving different proteins that would localize AMAPRs in the PSD as well as create a binding between the pre- and post-synaptic terminals (Sanhueza and Lisman, 2013). CaMKII is also important for the control of proteasome localization and activity. It regulates proteasome localization in the PSD and activation by phosphorylation of the Rpt6 proteasomal subunit (Bingol et al., 2010; Djakovic et al., 2009; Jarome et al., 2013). CaMKII also facilitates protein degradation by phosphorylation and consequent activation of CYLD. Activated CYLD will facilitate target protein degradation by removing K63-linked polyubiquitins from this protein (Thein et al., 2014). Finally, CaMKII might be involved in the synapse tagging mechanism in activated spines trapping plasticity related proteins diffusing to these spines. CaMKII could be the tag itself, regulate the migration of tag proteins to a spine or phosphorylate different proteins resulting in the tagging of a spine (Redondo et al., 2010). This is still to be determined.

Furthermore, Naskar et al. (2014) described a memory consolidation impairment due to inhibition of T305 autophosphorylation site of αCaMKII that could be rescued by proteasome inhibition. They also observed a decrease in the levels of the AMPA receptor subunit GluA1 due to CaMKII inhibition, and this decrease was rescued by proteasome inhibition (Naskar et al., 2014). Although the molecular pathways by which CaMKII

regulates consolidation are still not clear, the relevance of this kinase in memory consolidation has been shown by pharmacological inhibition of CaMKII immediately after training (Gholizadeh et al., 2013). However, the inhibitor of CaMKII used by Gholizadeh et al. (2013) was KN-62, which has been shown to also inhibit CaM activation of other members of the CaMK cascade (Enslen and Soderling, 1994; Mochizuki et al., 1993). CaMKII's relevance for memory consolidation has also been raised by studies with three different mouse models with CaMKII mutations. Heterozygous mice for a null mutation of αCaMKII have normal memory 1 to 3 days after training in two different hippocampus-dependent tasks, but their memory was impaired 10 to 50 days after training (Frankland et al., 2001). Point mutations of CaMKII's site that interacts with NMDAR's subunit GluN2B also resulted in consolidation impairment. These mutant animals can learn the water maze memory task, have a spatial memory 1h after training, but loose this memory when tested 1 day after training (Halt et al., 2012). Finally, animals with disruption of synaptic/dendritic translation of αCaMKII due to a mutation in a non-coding region of the messenger RNA (mRNA) of αCaMKII preventing the transport of this mRNA into dendrites show normal learning of MWM task, but impaired spatial memory in a probe trial 15 days after last training session. The same mutants also have normal cued and contextual fear memory when tested 30 minutes after conditioning, but not 1 day after. Similar results were observed in the object recognition task were animals have normal memory 1 hour after training, but impaired memory 1 day after training (Miller et al., 2002).

1.5.3 CaMKII and memory reconsolidation

Memory reconsolidation follows the retrieval of a memory and is important for the maintenance and further strengthening of this memory (Fukushima et al., 2014; Lee, 2008). Memory reconsolidation was shown to be a different process from memory consolidation due to their different biological substrates. Althought the two processes are dependent on gene expression, memory consolidation is dependent on hippocampal levels of BDNF but not Zif268 while the opposite is true for memory reconsolidation (Lee et al., 2004). Furthermore, Lee (2008) observed that the enhancement of a fear response due to a second training session, 24 hours after the first training session, was blocked by blocking hippocampal Zif268, showing that reconsolidation process was necessary for memory strengthening.

Retrieval of a memory can destabilize the memory, which involves proteasome dependent degradation of synaptic proteins, followed by restabilization of this memory, a protein synthesis-dependent process named reconsolidation (Kelly et al., 2003; Lee, 2008; Lee et al., 2004; Lee et al., 2008; Nader, 2003). Indeed pharmacological blocking of protein synthesis after retrieval impairs a previously consolidated memory (Nader et al., 2000). Blocking the proteasome system reverts the memory impairment effect of blocking protein synthesis (Lee et al., 2008), indicating that memory reconsolidation by protein synthesis is important to revert memory destabilization by protein degradation.

Nonetheless, blocking of protein degradation has also been shown to have a memory effect without blocking of protein synthesis. Pharmacological blocking of protein degradation immediately after retrieval session impairs memory maintenance (Artinian et al., 2008). Milton et al. (2013) have suggested that the regulation of these two processes are dissociated, with memory destabilization requiring NMDAR's subunit

GluN2B activation and memory restabilization the NMDAR's subunit GluN2A, both within the basolateral amygdala. CaMKII activity could be important for both, destabilization and reconsolidation. In terms of destabilization it has been observed that hippocampal inhibition of CaMKII post-retrieval impairs long term memories and that this effect is dependent of protein degradation (Da Silva et al., 2013). CaMKII's regulation of the proteasome system described previously in "1.5.2 CaMKII and memory consolidation" could explain Da Silva et al. (2013)'s observation. The identification of proteins targeted for degradation in this destabilization process is still poorly studied. First efforts have identified proteins involved in translational control and synaptic structure (Jarome et al., 2011; Lee et al., 2008).

Regarding synthesis of proteins, the immediate-early gene Zif268 is expressed by memory retrieval (Hall et al., 2001) and necessary for memory reconsolidation but not consolidation (Besnard et al., 2013; Lee, 2008; Lee et al., 2004). ERK seems to regulate the expression of Zif268 after late LTP (L-LTP) induction (Davis et al., 2000). As explained in session "1.5.2 CaMKII and memory consolidation" SynGAP's phosphorylation by CaMKII inhibits ERK's activation (Wang et al., 2013). This could be a mechanism by which CaMKII regulates ERK and Zif268 during reconsolidation. However, the relevance of ERK in memory reconsolidation is still a matter of debate. There have been conflicting findings regarding ERK's relevance for memory reconsolidation. Reports of both ERK-independence of memory reconsolidation (Lee and Hynds, 2013) and ERK-dependence (Besnard et al., 2013; Kelly et al., 2003) have been reported. A closer analysis of these three studies shows that Kelly et al. (2003) differ from the other two studies for not having used the CFC paradigm. Although both Besnard et al. (2013) and Lee and Hynds (2013) analysed the effects of inhibition of ERK activity in CFC memory reconsolidation

by treatment with mitogen-activated protein kinase kinase (MEK) inhibitors, treatment by Lee and Hynds (2013) was focused in the hippocampus while Besnard et al. (2013) used a systemic treatment.

Another molecular pathway that could explain how CaMKII may regulate Zif268-dependent memory reconsolidation is the IκB kinase (IKK)-Nuclear factor-κB(NF-κB) pathway. Such pathway has been shown to be important for memory reconsolidation in a Zif268-dependent matter (Lee and Hynds, 2013). Inhibition of CaMKII blocks activation NF-κB by depolarization, glutamate signaling or by basal activity (Meffert et al., 2003). In addition, the constitutively active CaMKII T286D mutant mice have increased NF-κB activation (Meffert et al., 2003). Furthermore, immunological studies revealed that CaMKII regulates NF-κB by a mechanism upstream of IKK (Maubach et al., 2013; Oruganti et al., 2011). Although the specific details of this mechanism are still unknown, it seems that the interaction with and phosphorylation of the signalling adaptor protein Bcl10 by CaMKII is an important step of this CaMKII regulation of NF-κB (Oruganti et al., 2011). Despite the mechanism by which CaMKII regulates Zif268 expression being unclear, transfection of dopaminergic cells with a vector containing a constitutively active form of CaMKII increased nuclear translocation of Zif268 (Takeuchi et al., 2002). Some kind of feedback response may exist between Zif268 and CaMKII, since it was observed that blocking of Zif268 expression with antisense oligodeoxynucleotide treatment down-regulates the expression and phosphorylation of CaMKII (Ganesh et al., 2012). Finally, the transcription factor CREB has also been suggested to be important for memory reconsolidation. Genetically modified animals expressing inducible CREB repressor have impaired memory reconsolidation in the MWM test (Kim et al., 2011). Additionally, the same mutant mice have impaired contextual fear memory

reconsolidation and immunohistochemical quantification of CREB showed an increase in hippocampal and amygdalar CREB expression after short memory retrieval (Mamiya et al., 2009). It has also been observed that artificial activation of CREB-expressing neurons in the amygdala induced a reconsolidation-like strengthening of the fear memory (Kim et al., 2014). As explained previously, βCaMKII phosphorylation of γCaMKII may have an indirect effect on CREB activation. Nevertheless, how CaMKII and all of these mechanisms and molecules interact during reconsolidation is still unclear.

1.5.4 CaMKII and memory extinction

Prolonged and repetitive retrieval of a memory in the absence of the US determine a gradual weakening of the memory, called memory extinction (Eisenberg et al., 2003; Pedreira and Maldonado, 2003; Suzuki et al., 2004). Memory extinction is considered to be a new learning process, or inhibitory learning, rather than memory erasure (Myers and Davis, 2007; Pape and Pare, 2010; Quirk and Mueller, 2008). Memory extinction can be identified by its behavioural peculiarities. An extinguished memory can be reinstated by a weak US that would not generate a memory in naïve animals (Rescorla and Heth, 1975; Trent et al., 2015) or by the presentation of an US in a different context (Corcoran et al., 2013). Extinguished memories also present spontaneous recovery, which is the reappearance of the CR in a memory test executed a long period after memory extinction (Archbold et al., 2010; Pavlov, 1927). Molecular analyses of memory extinction is an area of much interest for the development of treatments for post-traumatic stress disorder and addiction (Everitt, 2014; Fitzgerald et al., 2014; Pizzimenti and Lattal, 2015).

One candidate of a biological substrate of extinction is BDNF. Injection of BDNF in the hippocampal-infralimbic region and insular cortex have been shown to cause

memory extinction of conditioned fear memory, even in the absence of fear extinction training (Peters et al., 2010; Rodriguez-Serrano et al., 2014). BDNF has also been shown to be increased after memory extinction, as well as, BDNF mRNA levels (Xin et al., 2014). Furthermore, blocking of BDNF protein function or blocking of BDNF mRNA expression seem to impair memory extinction maintenance, inducing the recurrence of the CR (Radiske et al., 2015). Memory extinction seems to also be dependent of ERK activation. Activation of nuclear ERK was observed and required for contextual fear extinction (Chen et al., 2005; Ryu et al., 2008; Tronson et al., 2009).

Another molecule that seems to be important for memory extinction is the immediately early gene c-fos. C-fos expression has been shown to be induced by one session of CFC extinction training and decrease throughout the consecutive extinction session (Do-Monte et al., 2010). Furthermore, between-session extinction of auditory-cued fear memory was accompanied by an increase in the number of c-fos-positive neurons within the basolateral amygdala, the cingulate cortex, and the DG of mice (Plendl and Wotjak, 2010). Increase in c-fos expression seems to be important for consolidation of the extinguished memory (Santini et al., 2004) and to reduce spontaneous recovery of the memory (Herry and Mons, 2004). Nonetheless, after repeated fear extinction sessions c-fos levels are significantly reduced (Guedea et al., 2011; Tronson et al., 2009). In terms of the hippocampus, one prolonged (55 minutes) auditory extinction session induces an increase in c-fos expression in the DG, only if the session is performed in the trained context. Hippocampal sub-regions CA1 and CA3, on the other hand, presented increased c-fos expression after prolonged auditory extinction session in the trained or in a novel context (Knapska and Maren, 2009). More studies are necessary to better understand the function of c-fos and the changes in its

expression levels during and after memory extinction. Although ERK activation seems to cause an increase in c-fos production in the CA1 area of the hippocampus during memory consolidation (Gao et al., 2010), the activation of ERK seems to coordinate a decrease in CA1 c-fos levels after memory extinction (Guedea et al., 2011). Guedea et al. (2011) have proposed two separate molecular pathways involving ERK activation during memory formation and extinction, with decrease in c-fos expression and phosphorylation of the transcription factor c-Jun only in memory extinction. Furthermore, ERK activation has been observed to happen without consequent increase in c-fos expression (Cook et al., 1999; Nakakuki et al., 2010). Additionally, Cook et al. (1999) reported that ERK independency to c-fos expression was related to intra-cellular calcium levels, leaving the question of which molecule regulates c-fos expression during and after memory extinction.

The transcription factor CREB could also be a biological substrate of memory extinction. Indeed, mutant mice expressing an inducible CREB repressor have impaired memory extinction(Mamiya et al., 2009). In fact, Mamiya et al. (2009) reported an increase only in amygdalar CREB expression after long, extinction inducing, contextual fear memory retrieval, with no changes in hippocampal CREB expression. Looking at different time points after different moments of memory extinction protocol, in the MWM, Porte et al. (2011) observed an increase in hippocampal CREB levels, in the CA1 sub-region, 60 minutes after first memory retrieval. However after five memory retrieval sessions CA1's CREB level were normal at 60 minutes after the last test and decrease 15 minutes and 8 hours after the last test (Porte et al., 2011).

Regarding CaMKII and extinction, the partial reduction of CaMKII autophosphorylation in heterozygous T286A mutants impairs extinction of contextual

fear memory (Kimura et al., 2008). What is more, blocking of hippocampal CaMKII by KN-62 impairs memory extinction (Szapiro et al., 2003), as well as, inhibition by intra-hippocampal injection of the inhibitor autocamtide-2-related inhibitory peptide (AIP) blocks extinction enhancement by novelty exposure (de Carvalho Myskiw et al., 2014). However, the molecular pathways by which CaMKII might regulate or be regulated by other molecular substrates of extinction are still unknown.

1.6 CaMKII, hippocampus and CFC

The study of hippocampal molecules has led to the discovery of various molecular bases of memory formation and maintenance. For example the observation by Giese et al. (1998) that T286A mutant animals have impaired LTP formation was on hippocampal cells. The establishment of BDNF as an extinction inducing molecule by Peters et al. (2010) was based on injection of this molecule in the hippocampus. Pharmacological manipulation of hippocampal protein synthesis and protein degradation were the bases for the first report by Lee et al. (2008) of protein degradation as an important process for memory reconsolidation. Concerning CaMKII in the hippocampus it is known that neuronal activity can change the patterns of expression of αCaMKII and βCaMKII in to opposite directions, resulting in increased expression of the first and decrease expression of the second (Thiagarajan et al., 2002). It has also been established that spatial memory formation is a process dependent of the phosphorylation of hippocampal CaMKII at the T286 site (Cox et al., 2014; Giese et al., 1998). Additionally, it was also reported that blocking of CaMKII kinase activity in the hippocampus blocks spatial memory reconsolidation (Da Silva et al., 2013) and extinction enhancement due to novelty exposure (de Carvalho Myskiw et al., 2014).

Concerning the hippocampus-dependent task CFC, T286A mutant animals present CFC memory formation impairment and impaired short term memory when trained with one or three shocks. However if trained with five shocks T286A animals have normal CFC memory (Irvine et al., 2011; Irvine et al., 2005). Conditional deletion of αCaMKII from neural progenitor cells and their progeny, in adult mice, impaired CFC memory formation with one or three shocks (Arruda-Carvalho et al., 2014). Furthermore, heterozygotes T286A mice have CFC extinction impairment (Kimura et al., 2008) and blocking of hippocampal CaMKII kinase activity, by AIP injection, blocks novelty-induced CFC extinction memory enhancement (de Carvalho Myskiw et al., 2014).

1.7 CaMKII endogenous inhibitors

Given its importance it seems intuitive that CaMKII needs to be tightly regulated. However, the regulation is not well understood. Using yeast two-hybrid screen Chang *et al.* (2001; 1998) have described for the first time the existence of 2 endogenous CaMKII inhibitor proteins, CaMK2N1 and CaMK2N2, which are encoded by different genes. These authors have shown that CaMK2N1 and CaMK2N2 are 65% identical in their putative open-reading frame and 95% identical in its inhibitory domain. However CaMK2N2 but not CaMK2N1, requires constant presence of CaM to ligate to CaMKII (Chang et al., 2001). CaMK2N1 is a protein with 78 amino acids, expressed in the whole brain and with higher levels at the frontal cortex, hippocampus and inferior colliculus. On the other hand, CaMK2N2 has 79 amino acids, and it is expressed in testis and brain, where it has a higher expression in cerebellum and hindbrain. The two isoforms of CaMKII inhibitors are able to inhibit αCaMKII and βCaMKII, but their different pattern of expression throughout the brain and different dependence to CaM suggests a specificity

of target and function (Chang et al., 2001; Chang et al., 1998). As αCaMKII, CaMK2N1 is mainly expressed in forebrain and hippocampus whereas CaMK2N2 is enriched in cerebellum, as βCaMKII (Chang et al., 2001; Chang et al., 1998; Cheng et al., 2006; Erondu and Kennedy, 1985; Peng et al., 2004). But it is important to remember that CaMK2N2 is present throughout the brain, with considerable amounts in various regions like the hippocampus, prefrontal and piriform cortices, amygdala and thalamus (Chang et al., 2001; Radwanska et al., 2010). Hence the independency of function between CaMK2N2 and CaMK2N1 is still a matter of debate. Regarding their subcellular localization both isoforms have been shown to be present at the cell bodies and dendrites, but expression at synapses is still controversial since there have been claims both that it occurs in this region (Lucchesi et al., 2011; Saha et al., 2007; Saha et al., 2006) as that it does not (Chang et al., 1998; Radwanska et al., 2010). The articles that claimed that the inhibitors are not presented at synapses used CaMKIIN1 fused with fluorescent protein (Chang et al., 1998) or immunohistochemistry (Radwanska et al., 2010) to analyse by microscopy the localization of the inhibitors. On the other hand, articles that reported the presence of the inhibitors of CaMKII at synapses are based on western blot analyses of PSD fraction (Saha et al., 2007; Saha et al., 2006) or crude synaptosomal fraction (Lucchesi et al., 2011).

The endogenous inhibitors selectively block CaMKII CaM-dependent and autonomous enzymatic activity without significant inhibition of other kinases such as CaMKIV, PKC and PKA (Chang et al., 2001; Vest et al., 2007). Besides inhibiting CaMKII kinase activity, the inhibitors also block T305 autophosphorylation and the interaction with the NMADR subunit GluN2B, whilst they do not affect T286 autophosphorylation and decrease CaM dissociation from CaMKII (Vest et al., 2007). The inhibitory effects of

the CaMKII endogenous inhibitors seem to be related to the binding of these proteins to a hydrophobic region of the kinase domain, the T-site, and blocking by sterical inhibition the access of substrates to the S-site as well as binding of NMDAR to the T-site (Vest et al., 2007) (Figure 1.2-1). When Chang et al. (1998) described the first CaMKII endogenous inhibitor, CaMK2N1, they also produced by clonal expression a peptide based on 28 amino acids region of the CaMK2N1 situated near its COOH terminus. This peptide seems to have the same inhibitory effects as CaMK2N1 (Chang et al., 1998; Vest et al., 2007). It was called by the authors by the name of CaMKIINtide (Chang et al., 1998). CaMKIINtide was then optimized to shorter sequence of 21 amino acids (CN21) (amino acids 43-63 of CaMK2N1) (Vest et al., 2007) and later fused with trans-acting activator of transcription (tat) domain, increasing cell penetration, therefore been called tatCN21 (Buard et al., 2010; Vest et al., 2007). These two new generations of CaMK2N1-based inhibitors, as well as, CaMKIINtide are commonly used to study CaMKII functions (Buard et al., 2010; Chang et al., 1998; Gouet et al., 2012; Illario et al., 2003; Vest et al., 2007). Recently even shorten peptides have been proposed, which are thought to be more potent. These are the 19 amino acid version CN19 (Coultrap and Bayer, 2011) and the 17 amino acids CN17 (Gomez-Monterrey et al., 2013). It is worth noting that inhibition of CaMKII kinase activity only can also be achieved by substrate-based inhibitors, available under the name of AIP and AC3-I (Braun and Schulman, 1995b; Ishida et al., 1995; Pellicena and Schulman, 2014). These substrate based inhibitors are based on CaMKII substrates autocamtide-2 and autocamtide-3 (Hanson et al., 1989), but with a substitution of the threonine to an alanine (Braun and Schulman, 1995b; Ishida et al., 1995; Pellicena and Schulman, 2014). These inhibitors have also been used in studies involving CaMKII (Bian and Yu, 2015; Da Silva et al., 2013; Sag et al., 2014). However the specificity of the substrate-based inhibitors of CaMKII is still a matter of

debate. The inhibitor AC3-I has been shown to inhibit cellular actions of protein kinase D1 (PKD1) as well as those of CaMKII (Backs et al., 2009). Furthermore, AIP and AC3-I only block CaMKII kinase activity, there for they are unlike to have the same effects as the CaMKII endogenous inhibitors. The CaMK2N1-based inhibitors of CaMKII have been reported to inhibit CaMKII kinase activity specifically (Chang et al., 1998; Vest et al., 2007), as well as, block the binding to the NMDAR (Vest et al., 2007), reduce levels of CaMKII at the synapse (Sanhueza et al., 2011), inhibit T305 autophosphorylation (Vest et al., 2007), block binding to densin (Jiao et al., 2011), decrease clustering of this kinase in the dendrites (Tao-Cheng et al., 2013), induce the formation of aggregates of CaMKII and polyribosomes near the PSD and in dendrites (Tao-Cheng et al., 2013) and block binding to Cav2.1 calcium channels (Magupalli et al., 2013). Regarding electrophysiological effects, a study using the CaMK2N1-derived peptides showed persistent depression of synaptic strength and reversion of LTP saturation after application of these fragments in rat hippocampal slices (Gouet et al., 2012). Gouet et al. (2012) were unable to explain the molecular bases of these electrophysiological phenotypes, which seem to be independent of glutamatergic synaptic activity, calcium signalling, protein synthesis or proteasome-mediated degradation. They have also revealed that CaMKIINtide's depression of synaptic strength is not related to NMDAR-induced LTD, leaving the question of what is the molecular pathway of the electrophysiological effects observed in the presence of CaMK2N1-derived peptides. In accordance with this data, Wan et al. (2010) reported that the injection of CaMKIINtide in the invertebrate Lymnea, 24 hours after learning, blocks memory consolidation by a mechanism independent of NMDAR activation. Nevertheless, reversion of LTP saturation and reduced basal activity due to treatment with CaMK2N1-derived peptide has also been reported by Sanhueza et al. (2011) and Sanhueza et al. (2007). This

decrease in synaptic strength was only observed with concentrations of the inhibitor peptide necessary to disrupt the CaMKII/NMDAR complex, quantified by coimmunoprecipitation, but not at lower concentrations sufficient to inhibit CaMKII kinase activity only (Sanhueza et al., 2011). In fact, this observation is an important base for the model proposed by Sanhueza and Lisman (2013) that CaMKII must have a structural role in a big protein complex formed at the PSD that maintains NMDAR and AMPAR in this area of the synapse, explained previously in sub-chapter "1.5.1 Structural roles of CaMKII". Finally, the inhibition of CaMKII kinase activity by AIP treatment of hippocampal-neuronal culture and hippocampal slices impairs LTP induction and reduces NMDAR's sub-unit GluN2B levels in the synapse (Gardoni et al., 2009). As for AMPAR, treatment of hippocampal slices with CaMKIINtide blocks the increase in AMPAR conductance and in PSD GluA1 levels that occur in animals with benzodiazepine withdrawal (Shen et al., 2010). Similarly, CaMKIINtide injection in snail *Lymnaea stagnalis* significantly reduced the learning-induced elevation of GluA1 levels and impairs associative memory consolidation (Naskar et al., 2014). Additionally, this CaMKIINtide reduction of GluA1 levels was blocked by pharmacological inhibition of the proteasome system, indicating degradation of GluA1 induced by CaMKIINtide treatment (Naskar et al., 2014). Treating rat hippocampal slices with the peptide tatCN21, Buard et al. (2010) observed impairment of LTP induction but not impact on LTP maintenance. In the same study Buard et al. (2010) also observed that treatment with tatCN21 before contextual fear conditioning training session impaired memory acquisition, but treatment before the memory test had no effect on memory retrieval. As mentioned before, Wan et al. (2010) reported that the injection of CaMKIINtide 24 hours after learning blocked reward memory consolidation in *Lymnaea stagnalis*. As for memory reconsolidation, blocking of CaMKII kinase activity by intra-hippocampal AIP injection

blocked spatial memory reconsolidation and this effect can be reversed by proteasome inhibition (Da Silva et al., 2013). Intra-hippocampal AIP injection also blocked memory extinction enhancement due to novelty exposure (de Carvalho Myskiw et al., 2014). One should remember that AIP only blocks CaMKII kinase activity, therefore it is different from the endogenous inhibitors of CaMKII. To my knowledge, until this date no study has addressed the function of the endogenous inhibitors of CaMKII in memory reconsolidation or extinction. To summarize, the references presented above show that the inhibition of CaMKII by substrate-based or CaMK2N1-based peptides blocks memory formation, consolidation, reconsolidation, extinction, LTP induction, LTP saturation and synaptic basal activity. Although CaMKIIN1-based peptides have been widely used for studying CaMKII's function very little is known about the function of the endogenous inhibitors of CaMKII and when are they expressed in neurons. Two studies by our group have reported an increase in the expression of the endogenous CaMKII inhibitors after fear conditioning training, in brain regions related to the task (Lepicard et al., 2006; Radwanska et al., 2010). Whilst CaMK2N1 mRNA expression in the hippocampus was increased after contextual fear conditioning, CaMK2N2 mRNA levels were unchanged (Lepicard et al., 2006; Radwanska et al., 2010). The function of this CaMK2N1 increase is unknown. Buard et al. (2010) have observed that treatment with tatCN21, a CaMK2N1-based inhibitor, previous to CFC training session blocks memory formation, in mice. While CaMK2N1 mRNA levels were increased afer CFC training (Lepicard et al., 2006), CaMK2N2 protein levels were increased in the hippocampus after novel context exploration (Radwanska et al., 2010). Lucchesi et al. (2011) suggested that the inhibitors would play different roles in learning and memory. While CaMK2N1 would be important to block active CaMKII (autonomous CaMKII or CaMKII bound to the NMDA receptor), CaMK2N2 would regulate CaMKII activated by further stimuli. But further studies are

necessary to fully understand the CaMKIIN1 and CaMKIIN2 mechanism of action in learning and memory. Finally, the amyotrophic lateral sclerosis (ALS)-causing mutation, R495X, in the FUS gene (also known as FUS-ΔNLS) causes an overexpression of CaMK2N2, at mRNA and protein level, *in vitro* (Convertini et al., 2013). This observation further reinforces the importance of studying CaMKII endogenous inhibitors.

1.8 Aims & Objectives

Molecular mechanisms of learning and memory is an area of intensivy research as much is still unknown.

CaMKII has long been proposed to be a molecular basis of memory (Lisman, 1994; Lisman and Goldring, 1988a; Lisman and Goldring, 1988b). CaMKII role as an important molecule for learning and LTP induction has been well established (Giese et al., 1998), however it has been suggested that CaMKII is not necessary for memory (Buard et al., 2010) or LTP maintenance (Fujii et al., 2013; Lee et al., 2009; Lengyel et al., 2004). Nonetheless, the relevance of CaMKII for memory maintenance is still a matter of debate. Heterozygous T286A mice show impairment in contextual fear memory extinction (Kimura et al., 2008). Additionally, the CaMKII inhibitor CaMKIINtide blocks memory consolidation in the invertebrate Lymnea. Blocking of CaMKII kinase activity in the hippocampus by treatment with AIP blocks memory reconsolidation in mice (Da Silva et al., 2013). Therefore is still unclear if CaMKII is necessary for memory maintenance, but the use of CaMKII specific inhibitors might help clarifying this question.

Whilst it has been established that there are two endogenous CaMKII inhibitor proteins, and while corresponding peptides have been used to study CaMKII function in different systems, it remains unclear what are the physiological roles of the endogenous CaMKII inhibitors in learning and memory. Expression studies have suggested that the regulation of expression of these inhibitors has a role in regulating CaMKII during memory formation (Lepicard et al., 2006; Radwanska et al., 2010) but nothing has been shown in terms of memory maintenance. The aims of my PhD thesis were:

- To investigate the relevance of hippocampal endogenous inhibitors of CaMKII in learning and memory after contextual fear conditioning, by knocking down CaMKIIN1 and overexpressing CaMKIIN2 in the hippocampus of mice.
- To understand the molecular mechanisms involved in the behavioural phenotypes observed after manipulation of the expression of the inhibitors, by quantifying CaMKII total levels and phosphorylation, GluA1 total levels and phosphorylation, protein degradation and gene expression.

Chapter 2 Materials & Methods

2.1 Animals

For the experiments presented in this thesis we have used male, 10-12 week-old C57BL/6J mice. The subjects were housed in a 12-h light-dark cycle with food and water *ad libitum*. They were housed in groups of 3-5 animals *per* cage with sawdust, bedding material and a cardboard tube changed once a week. During the experiments animals were divided between the treatments so that each cage would include animals from all the treatments. During the collection of behavioural and molecular data the experimenter was blinded to the animal's treatment. All experiments were undertaken in accordance with the UK Animals (Scientific Procedures) Act 1986, under the project licence number 70/6772 and the personal lincence 70/24372.

2.2 Viral vector

During this work we used recombinant adeno-associated virus (rAAV), serotype 2/9, as vector for gene expression manipulation in the dorsal hippocampus of mice. rAAV vectors have been shown to be a safe and efficient way to transfect cells in the central nervous system (CNS), having a high vector production yield, expression onset of 1-2 weeks, expression duration of more than six months and very low chance of virus replication or gene insertion mutagenesis (Edry et al., 2011; Murlidharan et al., 2014; Ojala et al., 2015). The adeno-associated virus (AAV) is a non-enveloped, single stranded deoxyribonucleic acid (DNA) virus that can only replicate in the presence of a helper virus (Srivastava et al., 1983). Viral vectors used in this experiment had their viral genes *rep* (which encodes proteins important for replication of the genome) and *cap* (which encodes structural proteins of the virus) removed. The following three plasmids were constructed in our group by Dr. Walter Lucchesi and sent for viral production to Penn Vector Core (University of Pennsylvania, Philadelphia, PA, USA).

2.2.1 Control virus

Our control virus plasmid contained an ampicillin resistance gene for clonal selection. This gene encodes for the enzyme β-lactamase, which neutralises antibiotics in the penicillin group (Citri and Pollock, 1966). Our plasmid also contained 5' and 3' inverted terminal repeats (ITR), required by AAV for correct integration of the viral genetic information (Wang et al., 1995). Between the ITRs there was a scrambled sequence of a short hairpin ribonucleic acid (shRNA) without a target on mammal's genome. The expression of this shRNA was under the control of U6 promoter. The U6 promoter is a polymerase III promoter existent in human cell for the expression of small nuclear ribonucleic acid (RNA) (Paule and White, 2000). Besides being naturally

responsible for the expression of small RNAs, the U6 promoter has been shown to retain full activity following deletion of all sequences downstream of +1 position (Das et al., 1988) and it had previously been reported to be effective in the expression of different small RNAs (Ma et al., 2014b; Miyagishi and Taira, 2002; Paul et al., 2002). Additionally this plasmid carried a reporter gene expressing the fluorescent protein ZsGreen, under the synapsin 1 promoter. The synapsin 1 promoter is a neuron specific promoter driving high levels of expression (Kumar et al., 2015; McLean et al., 2014). We obtained the cloned synapsin 1 promoter region from Dr. Marco Peters, Darts Neuroscience, San Diego, USA. A schematic view of the plasmid is shown on Figure 2.2-1 and the sequence for all the inserts mentioned above can be found in Table 7.1-1 in the appendix. Viral production yielded a final titre of 1.2^{13} genome copies *per* millilitre (GC/mL).

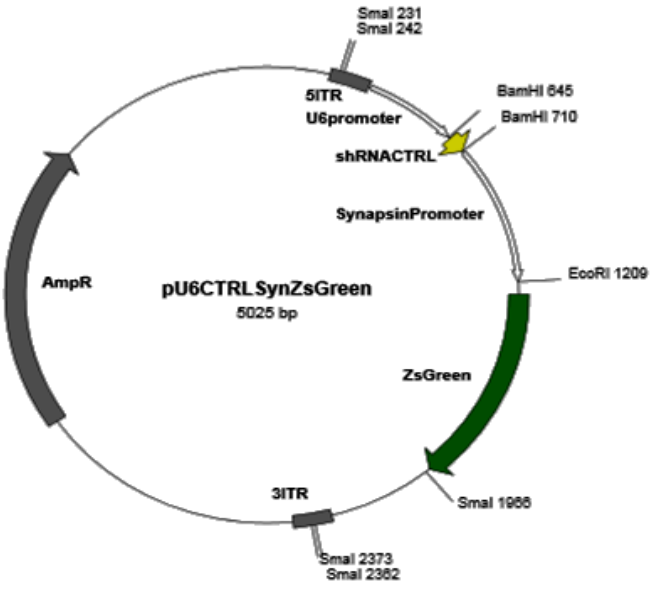

Figure 2.2-1 Schematic map of control plasmid
This circular plasmid was used to create the control rAAV. As the map show it contains a shRNA without any target in the genome (shRNACTRL), under the U6 promoter and ZsGreen reporter gene under the synapsin 1 promoter. The map also shows the location of the 5' and 3' ITR and the ampicillin resistance gene (AmpR). It also indicates some restriction enzymes that could be used for sub-cloning with this plasmid.

2.2.2 ShCaMK2N1 virus

In order to knock-down expression of the gene for the inhibitor CaMK2N1 a plasmid containing the shRNA sequence under the control of U6 promoter was used

(Appendix, Table 7.1-1). The plasmid also contained a ZsGreen gene under the control of synapsin 1 promoter, ampicillin resistance gene and the 5' and 3' ITRs needed (Figure 2.2-2). The resulting virus titre was 1.39^{13}GC/mL.

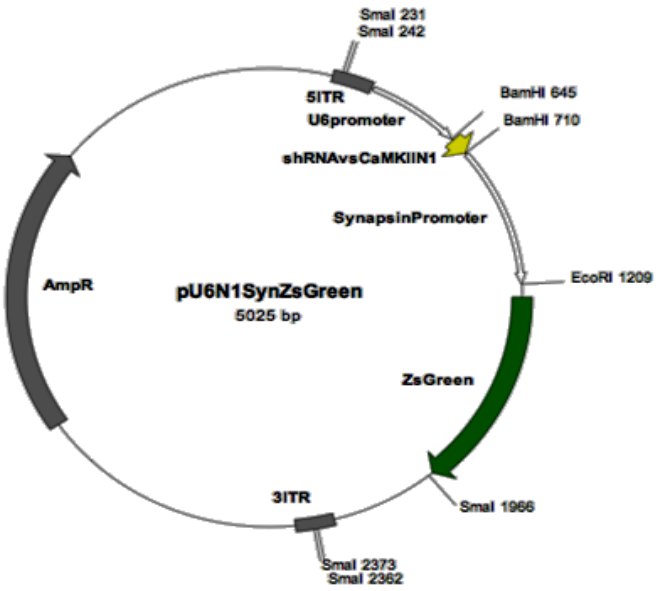

Figure 2.2-2 Schematic map of ShCaMK2N1 plasmid
This plasmid was used to generate a rAAV to knock-down CaMK2N1. As shown in the map it contains a sequence for a shRNA against CaMK2N1 mRNA (shRNAvsCaMKIIN1), under a U6 promoter and ZsGreen reporter gene under a synapsin promoter. It also shows the location of the 5' and 3' ITR and the ampicillin resistance gene (AmpR). It indicates as well some restriction enzymes that could be used for sub-cloning with this plasmid.

2.2.3 CaMK2N2 overexpression virus

To manipulate the expression of CaMK2N2 we have used a rAAV bicistronic vector based on the synapsin 1 promoter driving the independent expression of the human CaMK2N2 gene and the GFP reporter gene, both separated by an internal ribosome entry site (IRES) sequence (Appendix, Table 7.1-1). An IRES sequence carries internal ribosomal entry site region, which attracts the translation complex in the internal region creating multiple protein products from a single mRNA sequence (Jang et al., 1988; Pelletier and Sonenberg, 1988). The plasmid also contained the ITRs regions and the ampicillin resistance gene. A schematic view of the plasmid can be seen on Figure 2.2-3. The final titre of the suspension was 2.25^{13}GC/mL.

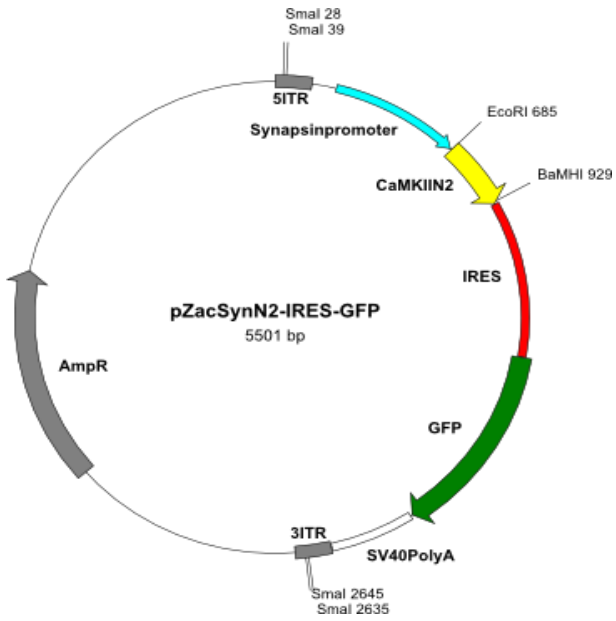

Figure 2.2-3 Schematic map of CaMK2N2 plasmid
This plasmid was constructed for gene manipulation of the expression of CaMK2N2. In light blue is the synapsin promoter that controls the bicistronic region composed by the CaMK2N2 gene, the IRES and the GFP reporter gene. The 5' and 3' ITR and the ampicillin resistance gene (AmpR) are also indicated in the scheme. It indicates as well restriction enzymes that could be used for sub-cloning with this plasmid.

2.3 Stereotaxic surgery

For the injection of virus solutions in dorsal hippocampus animals were subjected to stereotaxic surgery procedure as follow. Before the beginning of a surgery sheets, surgical gown, scissors and forcipes used during the surgery were autoclaved for asepsis. The experimentator washed his hand thoroughly with antimicrobial skin cleanser (chlorhexidine gluconate 4% w/v, Mölnlycke Health Care Limited, Bedfordshire, UK; 10009249). During the surgery an autoclaved gown, face mask, hair net and sterile gloves (Mölnlycke Health Care Limited, Bedfordshire, UK; 96270) were used to avoid contamination. The mouse was anaesthetized with a mixture of isoflurane (Abbott, Kent, UK; 05260-05) and O_2. The scalp was shaved and them the animal was positioned on a stereotaxic frame (David Kopf Instrument, Tujunga, CA; Model 963 Ultra Precise Small Animal Stereotaxic Instrument) over an electric heated blanket. At this moment animals received an intra-peritoneal injection of the analgesic Vetergesic (10mg/Kg)

(Buprenorphine - 0.3mg/mL – RECKITT BENCKISER Healthcare, Hull, UK) and the eyes of the animal were lubricated with lacri-lube (Allergan, Bucks, UK). The scalp was them cleaned with Povidone-Iodine solution (0.75%w/w – Animalcare, York, UK; XHG 012) for asepsis. A sterile disposable scalpel size 10 (Swann-Morton Limited, Sheffield, UK; 0501) was used to make an incision along the midline, exposing the skull. The skull was then cleaned with phosphate buffered saline (PBS) solution (Life Technologies, Paisley, UK; 10010-023) and bregma was identified. A round head micro drill steel burr of 0.5mm (Hager & Meisinger GmbH, Neuss, Germany; 19007-05) was used to make two holes on the skull over the hippocampus of both hemispheres. The coordinates used to determine the injection sites were anterior-posterior: +2.1mm; medio-lateral: ±1.5mm; dorso-ventral: -1.8mm from bregma. The total volume of injection was 0.75 µL of viral solution at a rate of 0.25µL/min, using a 2.5µL Hamilton syringe (Hamilton Medical, Reno, NV; 87942) and a microsyringe pump controller (UltraMicroPump (UMP3) with SYS-Micro4 Controller - World Precision Instruments, Sarasota, UK; UMP3-1). After injection the syringe was left without moving for 1 minute to allow for the injected solution to diffuse and was removed afterwards slowly from the brain (around 0.4mm every 15 seconds). Animals were then sutured (4-0 – FS-3 CONV – Mersilk® – Ethicon, Puerto Rico, USA; W501H), received a topical local anaesthesia (EMLA® cream 5% - Lidocaine 25mg/g – Prilocaine 25mg/g – Astra Zeneca, Luton, UK) and were taken to a recovery chamber with controlled temperature. After recovery from the anaesthesia the animal was housed again in his original cage. Mice were left two weeks without been subjected to any other procedure for recovery and also to wait for the genetic information of the virus to be expressed. During this period animals were checked for signs of pain and/or distress such as increasing loss of weight, self-inflicted injury, unattended fur, unattended nest and/or social isolation.

2.4 Contextual fear conditioning

To study learning and memory ability of the animals the CFC paradigm was used. The CFC is a Pavlovian conditioning task in which a neutral context [conditioned stimulus (CS)] is associated with an aversive US. After conditioning the CS evokes a learned fear response known as CR (Maren, 2008; Pavlov, 1926). This paradigm has previously been used successfully by our research group (Mizuno et al., 2012; Radwanska et al., 2010). All experiments were performed during light cycle. During the training session of the CFC the mouse was placed into the conditioning chamber (30.5 cm x 24.1 cm x 21.0 cm) (MedAssociates, Loughborough, UK; VFC-008-LP) containing a tissue wet with a small spray of 70% v/v ethanol (Sigma-Aldrich, St. Louis, MO, USA; 34935) under a metal grid on the floor in a soundproof box. The animal was exposed to the chamber context, the CS, for 40 seconds after which he received a foot shock of 0.7mA for 2 seconds, the US. The mouse was then left in the conditioning chamber for 30 seconds to distress. This was defined as the training session. To test for the memory of the association between the CS and the US animals were returned to the same conditioning chamber with the same 70% v/v ethanol olfactory stimulus and their behaviour was recorded for 5 minutes. Their freezing behaviour was used as CR and was scored every 5 seconds for 2 seconds. Freezing was positively scored if no movement other than respiratory movements were observed. To control the shock and record animals' behaviour VFreeze software was used (MedAssociates, Loughborough, UK). Scoring was done blind to treatment. In the first two experiments scoring was done twice by two different experimenters to test the precision of my scoring. Time points for the training and memory test sessions varied according to the aim of the experiment and are described in detail before every result.

2.5 Animal sacrifice and sample collection

2.5.1 Collection of samples for gene or protein expression

For analyses of gene or protein expression, after behavioural test animals were sacrificed by asphyxiation with increasing concentration of CO_2. Each animal was then decapitated, the skull was opened and the brain was immediately placed over a petri dish with absorbent paper soaked in saline solution, 0.9% w/v NaCl (VWR International, Radnor, PA, USA; BDH8014-500G), which was previously placed over ice. The brain was washed with saline solution and both hippocampi were dissected. Hippocampal samples were divided into dorsal and ventral as well as in right and left hippocampus. After dissection samples were kept in dry ice and subsequently stored at -80°C.

2.5.2 Collection of samples for fluorescent imaging

For samples used for fluorescent microscopy, animals were sacrificed by overdose with pentobarbital derived Euthatal® (Merial, Toulouse, France). Each animal received an intraperitoneal injection of Euthatal® in a dose of 1g/kg. Once analgesia was confirmed by pressing of tail and foot animals were attached by the back to a Styrofoam board. The animals rib cage was opened and a small cut was done at the heart in the right atrium. Simultaneously a needle connected by a Pumpsil® Platinum cured silicone tubing (3.2mm bore x 1.6mm wall thickness - Watson-Marlow, Falmouth, Cornwall, UK) to a 520U series IP31 pump (Watson-Marlow, Falmouth, Cornwall, UK) was introduced into the animal's heart in the left ventricle. The animal's blood was washed out by injection of approximately 10mL of PBS solution (Life Technologies, Paisley, UK; 10010-023) in a rate of approximately 10mL/min. Initial wash was followed by perfusion with 4% w/v paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO, USA; P6148) solution

(Table 7.3-1) at approximately 10mL/min for 8 minutes. Animal's brain was dissected and incubated overnight at 4°C. In the following day the brain was placed in 30% w/v sucrose (Calbiochem® - Merck Millipore, Darmstadt, Germany; 5737) solution diluted in PBS (Life Technologies, Paisley, UK; 10010-023) with 0.05% sodium azide (Sigma-Aldrich, St. Louis, MO, USA; 438456) at 4°C and stored until future analysis.

2.6 Fluorescence microscopy

As described in subchapter 2.2, the viral vectors used in this thesis had reporter genes that express one of the two fluorescent proteins, GFP or ZsGreen. Expression of these fluorescent proteins by transfection with all the three viral vectors used was accessed as a control for the surgery precision and viral effective transduction of genetic material. For that propose previously perfused samples were frozen over dry ice in optimal cutting temperature (OCT) compound (VWR international, Radnor, PA, USA; 25608-930) and sliced in a MicromHM 560 Cryostat-Series (Thermo Scientific, Waltham, MA, USA) in coronal sections of 40µm at a temperature of approximately -20°C. Sections were mounted in Superfrost® plus slides (VWR International, Radnor, PA, USA; 48311-703), covered with Vectashield® mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, USA; H-1200) and cover glass (22mm x 64mm)(VWR International, Radnor, PA, USA; 631-0880). Sections were visualized in a Zeiss AxioImager.Z1 fluorescent microscope (Zeiss, Oberkochen, Germany) with AxioVision software (Zeiss, Oberkochen, Germany) and a monochrome AxioCam MRm Rev.3 camera (Zeiss, Oberkochen, Germany). Images from GFP or ZsGrenn fluorescence were merged with DAPI florescence images, from the same

area, by image analyses software ImageJ (National Institute of Mental Health, Bethesda, MD, USA).

2.7 Gene expression

In order to assess CaMK2N1 and CaMK2N2 expression levels quantitative real-time polymerase chain reaction (RT-qPCR) was used. RT-qPCR is a combination between a polymerase chain reaction (PCR) and a fluorescent based reaction. In brief, the technique is based on transcription of the mRNA of a sample to complementary DNA (cDNA) by the enzyme reverse transcriptase. This step is followed by repetitive steps of amplification in high temperature with the enzyme taq polymerase and simultaneous detection and quantification of the amplified region of interest by use of fluorescent labelled oligonucleotides in the reaction (Higuchi et al., 1993). This technique has the unique ability to detect and simultaneously quantify the amplified gene of interest in real time with relatively good speed, accuracy and requirement of very small amounts of sample (Higuchi et al., 1993).

2.7.1 RNA extraction and quantification

All the procedures described in this section were executed using Gibco® DNase/RNase free water (Life Technologies, Paisley, UK; 10977035) and all equipment (pipettes, tube holders, gloves...) were clean with Ambiom® RNaseZap® (Life Technologies, Paisley, UK; AM9780). For this analysis right or left hippocampus of each mouse was picked randomly. The sample was then homogenized in 600µL of TRIzol® buffer (Life Technologies, Paisley, UK; 15596-026) using polytron homogenizer PowerGen 125 (Fisher Scientific International, Inc., Hampton, NH, USA; 12396727) 30 to 45 seconds. After 30 to 60 minutes incubation at room temperature (RT), 120µL of

chloroform (Sigma-Aldrich, St. Louis, MO, USA; 528730) was added to the sample and the sample was shacked for 30 seconds. Sample was left incubating for 10 to 30 min at RT before been centrifuged at 10,000 revolutions per minute (rpm) for 20 minutes at 4°C in table centrifuge (Heraeus™ Biofuge Fresco™ - Thermo Scientific, Waltham, MA, USA; 75002420). Aqueous phase was then transfered to a clean tube and RNA was precipitated by incubating the solution for 15 to 30 minutes at RT with 300µL of isopropanol (VWR International, Radnor, PA, USA; 700002-600). Following incubation the sample was centrifuged again at 10,000rpm for 20 minutes at 4°C and supernatant was discharged. Precipitated pellet was washed with 600µL of 75% v/v ethanol (Sigma-Aldrich, St. Louis, MO, USA; 34935) and centrifuged at 10,000rpm for 5 minutes at 4°C. Supernatant was discharged again followed by a second 10,000rpm for 5 minutes at 4°C centrifugation step. Any left supernatant was disposed and the precipitated pellet was diluted in 30µL of Gibco® nuclease-free water (Life Technologies, Paisley, UK; 10977035). Sample was quickly vortexed and left to stand on ice for 1 hour. After this last incubation each sample was quantified and their 260nm/280nm ratio was obtain by spectrophotometer analyses using NanoDrop ND1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA, a ratio of 2.0 was considered to be approximately 100% purity. All the samples (approximately 200ng of each) were submitted to electrophoresis for 40-45 minutes at 85V in a 1.2% w/v agarose (Life Technologies, Paisley, UK; 16500-500) gels and visualised in BioSpectrum® imaging system (UVP, Upland, CA, USA) to check for the presence of the 18S and 28S ribosomal RNA fractions as shown in Figure 2.7-1.

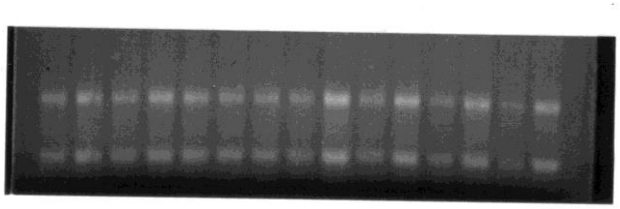

Figure 2.7-1 Example of 18S and 28S ribosomal RNA fractions
Figure shows an example of the separation and visualisation of the ribosomal RNA fractions of 18S and 28S. All RNA extracted samples were subjected to this analysis to confirm the success of the RNA extraction. Samples were run in 1.2% w/v agarose gel for 40-45 minutes at 85V. Visualization of the RNA fractions was achieved true mixture with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA; E1510) and visualisation at BioSpectrum® Imaging System running VisionWorks® LS Image Acquisition and Analysis Software (UVP, Upland, CA, USA).

2.7.2 First Strand cDNA Synthesis

Reverse transcription of RNA to cDNA was performed using SuperScript® II Reverse Transcriptase (Life Technologies, Paisley, UK; 18064-014) according to published protocol(Lepicard et al., 2006). In brief, 1.3µg of sample RNA template was diluted in nuclease free water (Life Technologies, Paisley, UK; 10977035) to a final volume of 10.5µL and mixed with 1µL of oligo(dT)$_{12\text{-}18}$ primer (Life Technologies, Paisley, UK; 18418-012), which targeted polyA mRNA. The solution was then incubated for 10 minutes at 70°C on Applied Biosystems® GeneAmp® PCR System 9700 (Life Technologies, Paisley, UK). The resulting solution was mixed with 10mM deoxynucleotide (dNTP) mix (Qiagen, Hilden, Germany; 201900), 0.1M dithiothreitol (DTT) (Life Technologies, Paisley, UK; D-1532), RNasin® Inhibitor (Promega, Fitchburg, WI, USA; N2115), SuperScript® II Reverse Transcriptase (Life Technologies, Paisley, UK; 18064-014) and 5x first strand buffer (Life Technologies, Paisley, UK; 18064-014) as described in Appendix Table 7.3-2 . The mixture was incubated at 42°C for 50 minutes before inactivation step of 10 minutes at 70°C using an Applied Biosystems® GeneAmp® PCR System 9700 (Life Technologies, Paisley, UK). Reactions were diluted 1 in 10 with nuclease-free water to a final volume of 180µl and stored at -20°C prior to use as template for PCR amplification reactions.

2.7.3 RT-qPCR primer and cDNA dilution

Primers complementary to CaMK2N1, CaMK2N2 and the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) were previously designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) and tested for specificity by sequencing of the amplification product as described in Lepicard et al. (2006). Primers' sequences can be seen in Table 7.2-1 of the Appendix. Optimum dilution for each primer was determined by a bigger difference between a random control sample and a blank solution in the number of cycles needed to reach exhaustion of the reaction. The concentration chosen for each primer is presented in Table 7.2-1. HPRT was selected as the housekeeping gene due to his higher efficiency of amplification with a random control cDNA sample as compared to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The sequence used as primer for HPRT (Table 7.2-1) was also compared in terms of efficiency with a different primer sequence for HPRT. Dilution of the cDNA samples was determined by equal amplification efficiencies of both the template and HPRT throughout a range of cDNA dilutions. The best dilution for the samples was calculated by doing a linear regression with the logarithmic value of the different sample dilutions and the difference between the cycles of housekeeping gene and the gene of interest. Different points of the obtained line were taken out of the regression with the aim of decreasing the "a" value of the equation describing the line. Optimum sample dilution was defined as a middle point in the line which the equation describing it had an "a" value smaller then 0.1. The best dilutions for the cDNA samples using CaMK2N2 or CaMK2N1 primers usually varied between 500X and 1000X dilution.

2.7.4 RT-qPCR

RT-qPCR was performed in 0.2mL non-skirted low profile 96-well PCR plate (Life Technologies, Paisley, UK; AB-0700) capped with an ultra-clear qPCR caps (Life Technologies, Paisley, UK; AB-0866). Each well contained 5µL of the cDNA template solution and 15µL of RT-qPCR solution, composed of forward and reverse primers, nuclease free water and the fluorescent marker 2x qPCR MasterMix with SYBRGreen and ROX (PrimerDesign Ltd., Hants, UK; Precision-R) with concentrations of each reagent as described in Table 7.3-3 of the Appendix. The amplification of the PCR product was continuously measured by Chromo4™, real-time PCR detector using a DNA Engine® (Bio-Rad, Hemel Hempstead, UK; CFB-3240) during 45 cycles. The reaction started with one step of 95°C for 10 minutes, followed by 45 cycles of 20 seconds at 95°C, 20 seconds at 60°C and 20 seconds at 72°C. The number of cycles needed to reach exhaustion of the reaction, as well as, the melting points of paired cDNA strands made in the reactions was calculated for each well with the analysis software Opticon Monitor, version 3.1.32 (Bio-Rad, Hemel Hempstead, UK). Every sample was performed in triplicates, and mean threshold cycle (CT) was determined. The CT value of each sample was normalized to the CT value of HPRT and calibrated to the CT value in the control group. This was achieved by subtracting the CT value of the housekeeping gene from de CT value of CaMK2N1 or CaMK2N2, ΔCt. The ΔCt of each sample was them transformed in Power (2, - ΔCt). The average power for the control group was calculated, and the average and error obtain for each group was divided for the average of the control group.

2.8 Protein analysis

To determine the regulation and expression of different proteins western blotting was used. This technique is sensitive to proteins with low abundance and relatively easy

to adapt to the quantification of different proteins (Kurien and Scofield, 2015). In brief, proteins were extracted from the tissue, their tertiary and secondary structure were disrupted, proteins were coated with negative charges, separated by molecular weight in an electrical field, transferred to a membrane and detected with specific antibodies (Renart et al., 1979; Towbin et al., 1979).

2.8.1 Protein extraction and fraction separation

For protein extraction the right and left hippocampus of each mouse were combined into one sample. This sample was homogenized in 300µL of homogenization buffer described on Table 7.3-4 of the Appendix. The homogenization buffer contained protease and phosphatase inhibitors (Table 7.3-4) but no detergent, to allow collection of different fractions of the cells. Homogenization was made on ice and by douncing by hand 20 times in a borosilicate glass tissue grinder (Kimble Chase, Vineland, NJ, USA; K885300/0002). The resulting solution was centrifuged at 4°C for 5 min at 2000rpm in a table centrifuge (Heraeus™ Biofuge Fresco™ - Thermo Scientific, Waltham, MA, USA; 75002420). The pellet was separated and re-diluted in 100µL of homogenization buffer. This fraction of the sample was named P1 and used for analysis of nuclear proteins. The supernatant fraction was collected and centrifuged at 13.000rpm for 15 minutes at 4°C. The resulting supernatant was collected and was identified as fraction S2, which is enriched for cytosolic protein. The pellet was re-diluted in 100µL of homogenization buffer. This solution, the P2 fraction, is a crude synaptosomal fraction. Similar fraction separation protocols can be found in Drakulic et al. (2013), Stanojlovic et al. (2015) and Han et al. (2014). P1, S2 and P2 were frozen at -20°C for further analyses. It is important to notice that animals' asphyxiation with CO_2, used to sacrifice the animals, can cause CaMKII precipitation as a side effect. If that was the case we

might had lost some CaMKII in the first fraction, P1, which was not analysed for CaMKII content. However this effect was not enough to make the quantification of CaMKII in the S2 and P2 fraction unfeasible, therefore it was ignored.

2.8.2 Protein quantification

Total protein concentration of each sample was determined with the Pierce™ BCA Protein Assay Kit (Life Technologies, Paisley, UK; 23225). In brief, the sample was diluted 10X with ultrapure water and 25µL of this solution was plated on 96-well Corning® Costar® cell culture plates (Sigma-Aldrich, St. Louis, MO, USA; CLS3997) with 200µL of 50:1 mix of reagent A and reagent B from Pierce™ BCA Protein Assay Kit (Life Technologies, Paisley, UK; 23225). The detection of the proteins in this kit was based on a colorimetric change in the solution due to a valence change in copper as a result of the reaction between copper and these proteins. A standard curve of bovine serum albumin (BSA), going from 2000µg of protein to 0µg, was also plated and mixed with reagent A and reagent B. After 30 minutes incubation at 37°C the plate was read at DTX 880 Multimode Detector (Beckman coulter®, Brea, CA, USA) with absorbance at 595nm. Linear regression between the observed absorbance of the standards with their known concentrations gave the equation that determined each sample's concentration. A regression would only be used for determine samples' concentration if the observed R^2 value was above 0.98.

2.8.3 Western blot

2.8.3.1 Gel electrophoresis and transfer

Samples were diluted in ultrapure water and 4X sample buffer (Table 7.3-5) as so that every sample had in the same volume the same quantatie of total protein. Total protein quantities used were 20µg or 50µg, depending on the protein to be analysed (for more details see Table 7.4-1). The samples were heated to 95°C for 5 to 10 minutes. Immediately after, the samples were placed on ice for at least 5 minutes. After a quick spin-down of the samples they were then blotted in 18-wells Criterion TGX AnykD gel (Bio-Rad Laboratories, Hemel Hempstead, UK; 567-1124) or 18-wells Criterion TGX Gel, 4-15% gel (Bio-Rad Laboratories, Hemel Hempstead, UK; 567-1084). If the number of samples surpassed the number of wells in the gel more than one gel was used, but the control samples were evenly distributed through the gels. In the first and last well of each gel 7µL of Spectra™ multicolour broad range protein ladder (Life Technologies, Paisley, UK; 26634) were added. Loaded gels were run on running buffer (Table 7.3-6) in two different conditions. For samples from P1 fraction the samples were run in the gel for approximately 15 minutes at 120V and 1 hour and 20 minutes at 100V. For P2 and S2 fractions the samples were run for approximately 1 hour and 50 minutes at 80V. After separation of proteins by size the proteins in the gel were transferred to a nitrocellulose membrane (Immun-Blot® PVDF Membrane – Bio-Rad Laboratories, Hemel Hempstead, UK; 162-0177) previously activated in methanol (Fisher Scientific International, Inc., Hampton, NH, USA; A4124) for 5 minutes. The gel and the membrane were placed above each other and the proteins were transferred at 150V for 1 hour and 15 minutes on transfer buffer (Table 7.3-7). Afterwards the membrane was washed with 30mL of tris-buffered saline and 0.05% v/v Tween™ 20 (Fisher Scientific International, Inc., Hampton,

NH, USA; BP337100) (TBST) (see Table 7.3-8 for tris-buffered saline (TBS) composition) for 10 minutes at 45 oscillations *per* minute (osc/min) on a see-saw rockers SSL4 shaker (Bibby Scientific Limited, Staffordshire, UK) and used for different immunostaining procedures.

2.8.3.2 Immunostaining and detection

After the membrane was washed it was blocked by shacking at 25 osc/min for 1 hour with 30mL of blocking buffer 5% w/v milk, composed of 1.5g of skim milk powder (Merck Millipore, Darmstadt, Germany; 115363) in 30mL of TBST 0.05% v/v. Membrane was subsequently incubated overnight at 4°C shacking at 55rpm in a Scientific C10 platform shaker (New Brunswick Scientific Co., Inc., Edison, NJ, USA) with 10-30mL of primary antibody solution. Primary antibody solution contained blocking buffer 5% w/v, sodium azide 0.05% w/v and the primary antibody of interest. A detailed list of the primary antibodies used in this thesis can be found in Table 7.4-1 of the Appendix. At the following day the membrane was washed three times with TBST at 45 osc/min at room temperature. Thereafter, the membrane was incubated at room temperature for 2 hours at a constant shack of 25 osc/min with secondary antibody solution composed of blocking buffer 5% w/v and the correct secondary antibody to bind the primary antibody of interest with the according dilution as described on Table 7.4-1 of the Appendix. This incubation was followed by three rounds of washing with 30mL of TBST for 10 minutes each. For quantification of pRPT6 the antibody used was kindly donated by Professor Fred J. Helmstetter (Department of Psychology, University of Wisconsin-Milwaukee, WI, USA) and is listed on Table 7.4-1. To fit our protocol to the previous used with this antibody, as described in Jarome et al. (2013), the TBST solution used was 0.1% v/v and the primary antibody was diluted in TBST 0.1% v/v with

3% w/v BSA. The primary antibody for p-c-jun was also diluted in 3% w/v BSA. For detection of all the secondary antibodies the Pierce™ ECL western blotting substrate (Thermo Scientific, Waltham, MA, USA) was used. In brief, the staining of the secondary antibody was based on the reaction between the horseradish peroxidise attached to the secondary antibody and enhanced luminol-based chemioluminescent substrate provided by the kit. The membrane was incubated for 5 minutes at 25 osc/min with 10mL of reagent 1 and 10mL of reagent 2. After incubation the membrane was quickly allocated inside a light proof recipient and taken to a dark room where a light sensitive Amersham Hyperfilm™ ECL™ (GE healthcare, Little Chalfont, UK; 28-9068-36) was exposed to it for detection of the chemioluminescence. Exposition time varied between the different antibodies. Quantification of bands was done after exposure in the linear range. The images obtained were analysed in ImageJ software (National Institute of Mental Health, Bethesda, MD, USA) for quantification of intensity of each band, by integration of the area of the graph obtain from the threshold analyses. For normalization the results of every protein of interest were divided by the results from the same membrane, after stripping, of a housekeeping protein, or the total protein of interest, if the antibody of interest was targeting a phosphorylated protein. Example membranes can be seen on Appendix “7.5 Example images of western blot membranes”. The results were also calibrated to the control value by multiplying the value of each sample by a factor created from dividing 1 to the average of the control samples in each gel separately. The average of each group was presented as arbitrary units (A.U.).

2.8.3.3 Membrane stripping

For reuse of the membranes with a different primary antibody the following stripping protocol was used. Membrane was washed for 10 minutes at 45osc/min with 30mL of TBST 0.05% v/v, followed by an incubation of 1 hour at 45osc/min with 20mL of buffer A and 200µL of buffer B from western blot stripping buffer (Santa Cruz Biotechnology Inc., Dallas, TX, USA; sc-281698). After this incubation the membrane was washed with 30mL of TBS for 5 minutes at 45osc/min. Thereafter the membrane was washed again with 3 rounds of 30mL of TBST 0.05% v/v for 10 minutes at 45osc/min. One final washed of 5 minutes at 45osc/min with 30mL of TBS was done before the membrane was blocked and used again for staining for a different protein.

2.9 Statistical analysis

Data was checked for normal distribution by Q-Q plot analysis, Kolmogorov-Smirnov and/or Shapiro-Wilk normality test. In case of non-normal distribution outliers were excluded from experiment as a normalization strategy. Outliers were defined as values that fall more than 1.5 times the interquartile range above the third quartile or below the first quartile. The only data remained not normally distributed after this normalization strategy was phosphorylation of CREB, so it was analysed with the non-parametric Kruskal-Wallis test. Animals treated with shCaMK2N1 virus were also excluded from the experiment if the RT-qPCR analysis showed that the level of expression of CaMK2N1 was outlier in his group. This was considered to be an indication of treatment failure. The same principal was applied for animals treated with CaMK2N2 virus but with RT-qPCR analysis of CaMK2N2 levels of expression. One-way analysis of variance (ANOVA) was used for analysis of dependent variables if the experiment had only one independent variable. In experiments with two independent variables,

dependent variables were analysed by two-way ANOVA, with repeated measures if necessary (for example when comparing behavioural results from first and second memory test). Student-Newman-Keuls (SNK) test was used when necessary as planned comparisons. P values bellow 0.05 were considered to be significant in every analysis. Data was presented in scatterplot graphs where every dot represented the value of one animal and a line indicated the average of the group. The use of such graphical representation has been recommended by Weissgerber et al. (2015) as more representative of the data and giving more information for a more complete analyses. Non-normally distributed data was presented in box-and-whisker plot with group median value, lower quartile and upper quartile represented in the box and lowest and higher values indicated by the whisker. This type of graph representation is the most indicated for non-normally distributed data (Weissgerber et al., 2015).

Chapter 3 Results with shCaMK2N1 virus

3.1 Fluorescent images after viral vector transfection

Images presented on Figure 3.1-1 and Figure 3.1-2 were taken from the brain of animals sacrificed 2 weeks after viral solution injection. As can be observed the fluorescence is restricted to the hippocampus, indicating that the virus effectively transfected neuronal cells only in the hippocampus.

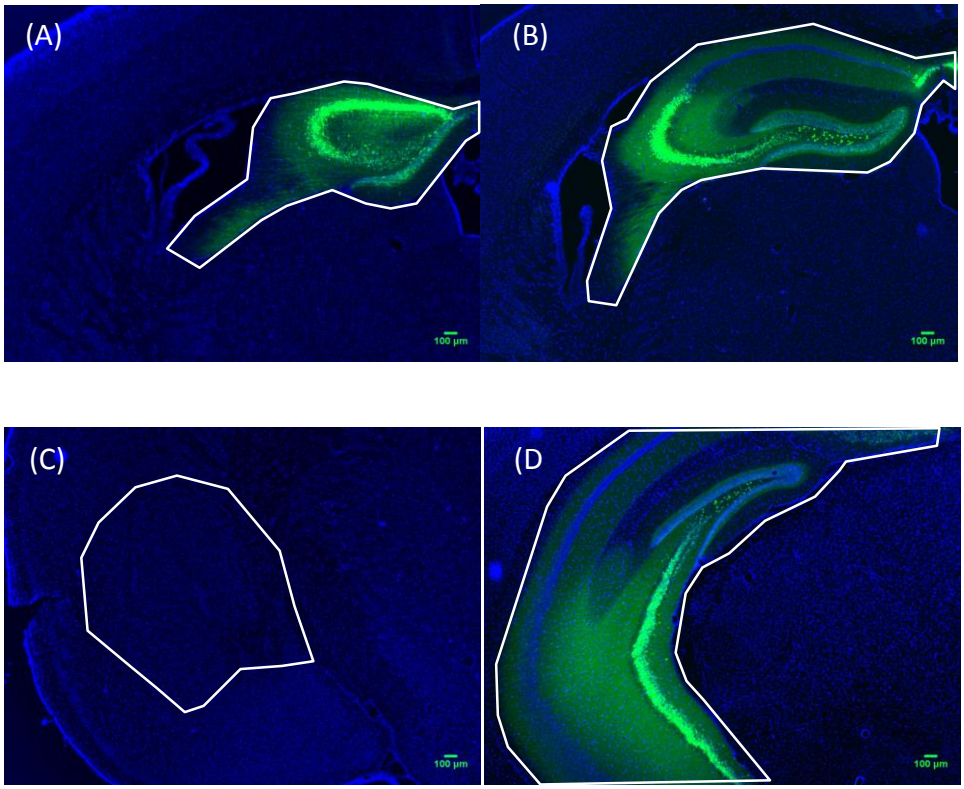

Figure 3.1-1 Fluorescent images for control virus
Pictures of a mouse brain injected with the control viral solution. Panels A to C show a sequence of images from coronal sections of the same hippocampus in an anterior-posterior order. The hippocampus is outlined inside the white line. Panel D shows an image of the amygdala of the same animal where one can notice the lack of ZsGreen fluorescence. The amygdala is outlined inside the white line. All the pictures show DAPI nuclear staining in blue and ZsGreen fluorescence in green. Pictures were taken with a 2.5X objective and the scale can be seen on the bottom right corner.

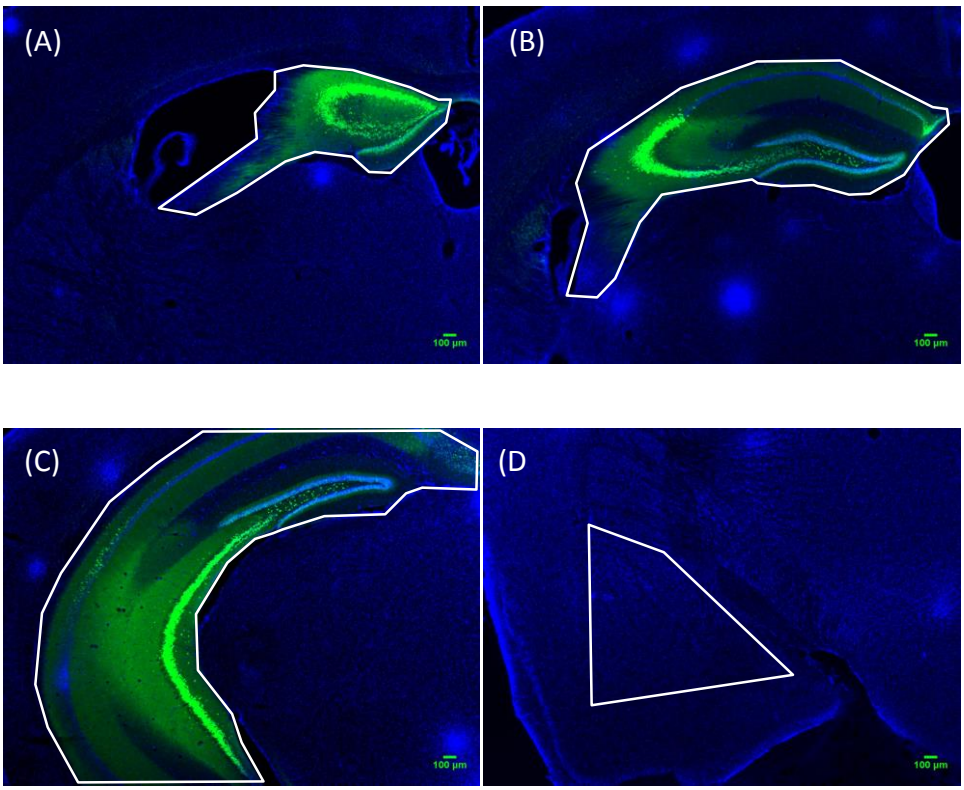

Figure 3.1-2 Fluorescent images for shCaMK2N1 virus
Pictures of a mouse brain injected with shCaMK2N1 viral solution. Panels A to C show a sequence of images from coronal sections of the same hippocampus in an anterior-posterior order. The hippocampus is outlined inside the white line. Panel D shows an image of the amygdala of the same animal where one can notice the lack of ZsGreen fluorescence. The amygdala is outlined inside the white line. All the pictures show DAPI nuclear staining in blue and ZsGreen fluorescence in green. Pictures were taken with a 2.5X objective and the scale can be seen on the bottom right corner.

3.2 Virus effect in dorsal and ventral hippocampus

Knowing that the transfection of the hippocampus was working we decided to verify the efficiency of the knockdown treatment. For that either control or shCaMK2N1 viral solution was injected into the dorsal hippocampus. After 2 weeks the animals were sacrificed and dorsal and ventral hippocampus were collected separately. A qPCR analysis examined the level of CaMK2N1 mRNA expression (Figure 3.2-1). Two-way ANOVA test showed a significant effect of virus treatment ($F_{1,22}$=13.1; P=0.002) and significant difference between regions ($F_{1,22}$=37.1; P<0.001), with no interaction effect ($F_{1,22}$=1.9; P=0.184). SNK planned comparisons showed that ventral hippocampus had higher levels of CaMK2N1 mRNA than dorsal hippocampus within the shControl group (q=4.5; P=0.005) and within shCaMK2N1 group (q=7.8; P<0.001). SNK test also indicated

that the virus treatment resulted in a significant decrease of mRNA levels of CaMK2N1 within dorsal hippocampus (q=5.0; P=0.003) but not ventral hippocampus (q=2.2; P=0.131). Based on this last result we have decided to work only with dorsal hippocampal samples. This means that all results from hippocampal analyses presented from now on in this thesis are only based on dorsal hippocampus samples.

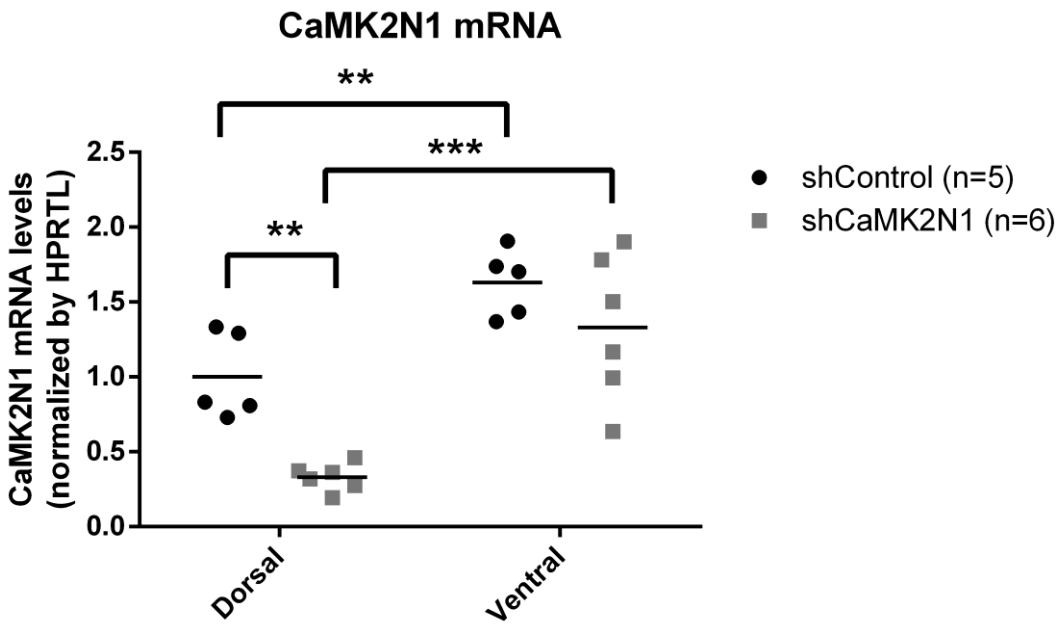

Figure 3.2-1 CaMK2N1 mRNA in dorsal and ventral hippocampus
Quantitative analysis of CaMK2N1 mRNA levels for dorsal and ventral hippocampus. All results are normalized to the average of the dorsal hippocampus of shControl group.** P<0.01; *** P<0.001

3.3 Effect of CaMK2N1 knockdown on memory maintenance

Figure 3.3-1 shows the experimental design used to study the effect of hippocampal CaMK2N1 knockdown in the CFC paradigm. Briefly, animals were injected intra-hippocampally either with shCaMK2N1 virus solution or shControl virus solution. Two weeks later animals were placed in the conditioning chamber and received a mild foot shock, the US. Animals' memory of the context was tested by re-exposure to the conditioning chamber without US for 5 minutes, 24 hours and 4 days after the training session.

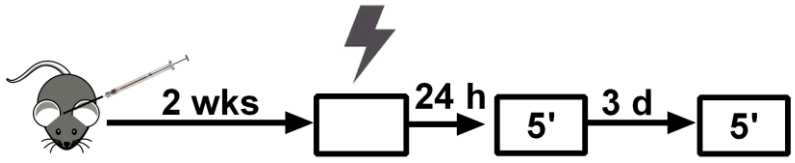

Figure 3.3-1 Experimental design CaMK2N1 knockdown
The figure shows the experimental design for the first test done on the effect of CaMK2N1 knockdown in the animals' freezing score at the CFC paradigm. Animals were first injected with shControl or shCaMK2N1 virus solution. Two weeks later they were trained with one shock of 0.7 mA. Animals were then re-exposed to the context in two memory tests of 5 minutes each. The tests occurred 24 hours and 4 days after training. Animals were sacrificed two hours after second memory test.

Animals freezing score during the two memory tests can be seen in Figure 3.3-2. Two-way ANOVA showed a significant effect of the two consecutive memory tests ($F_{1,24}$=9.0; P=0.006) with no effect of virus treatment alone ($F_{1,24}$=1.4; P=0.234) but a significant effect of interaction ($F_{1,24}$=8.1; P=0.009). SNK planned comparisons revealed a significant difference between the freezing score of animals from shControl group and shCaMK2N1 group in the 4 days memory test (q=3.4 ; P=0.019) but no difference in the 24 hours memory test (q=0.4 ; P=0.769). This result indicated that CaMK2N1 knockdown has an effect on memory maintenance rather than on memory formation. A significant difference between first and second memory test was observed on animals from shCaMK2N1 group (q=3.7 ; P=0.012) but not on animals from shControl group (q=0.1 ; P=0.936).

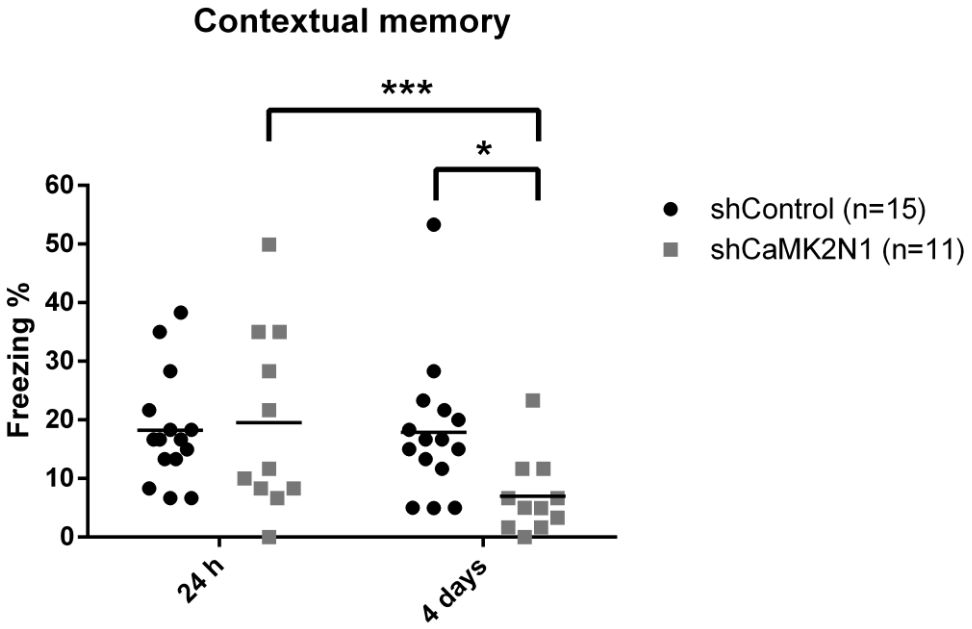

Figure 3.3-2 Freezing scores from experiment with CaMK2N1 knockdown
The graph above shows the average of freezing scores of animals from shControl and shCaMK2N1 groups, in the first (24h) and second (4 days) memory tests. Freezing scores are presented in the ordinate axis as percentage of freezing time in the 5 minutes of test. *P<0.05; ***P<0.001

RT-qPCR analyses of dorsal hippocampus confirmed an effective knockdown of CaMK2N1 (Figure 3.3-3). One-way ANOVA showed a significant decrease of CaMK2N1 mRNA levels in shCaMK2N1 group ($F_{1,25}$=17.7; P<0.001). Interestingly, CaMK2N2 mRNA levels were significantly increased in animals from shCaMK2N1 group ($F_{1,25}$=13.3; P=0.001) (Figure 3.3-4). This increased expression of CaMK2N2 could be interpreted as a compensation for the knocking-down of CaMK2N1 and it was studied in more detail in Chapter 4.

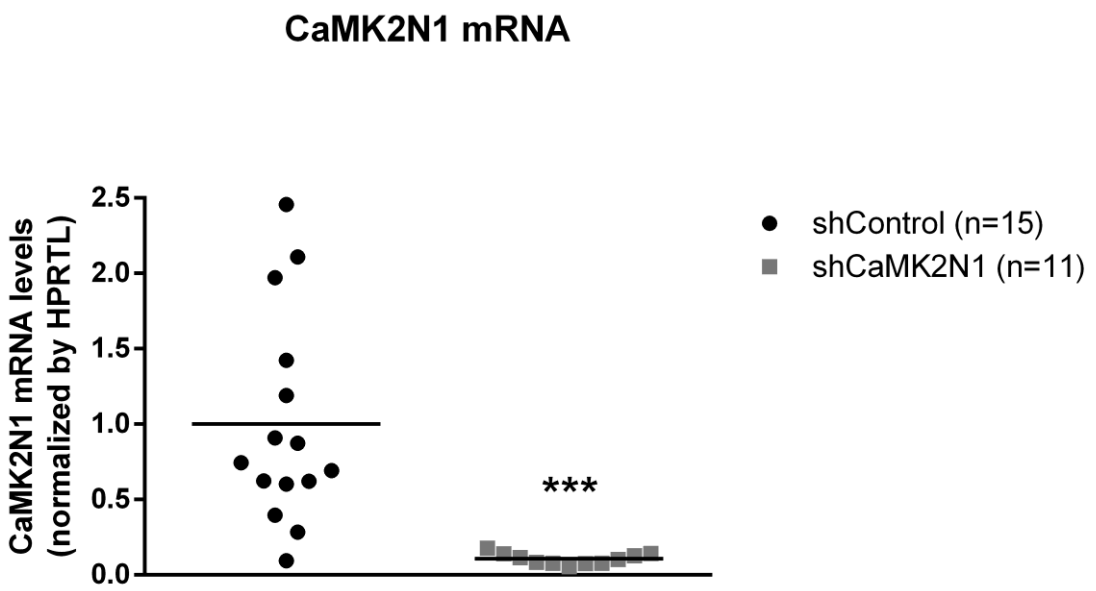

Figure 3.3-3 CaMK2N1 mRNA levels from experiment with CaMK2N1 knockdown
Quantitative analysis of the levels of CaMK2N1 mRNA. CaMK2N1 mRNA levels were normalized by HPRTL mRNA levels. All results were standardized to shControl group average. *** P<0.001

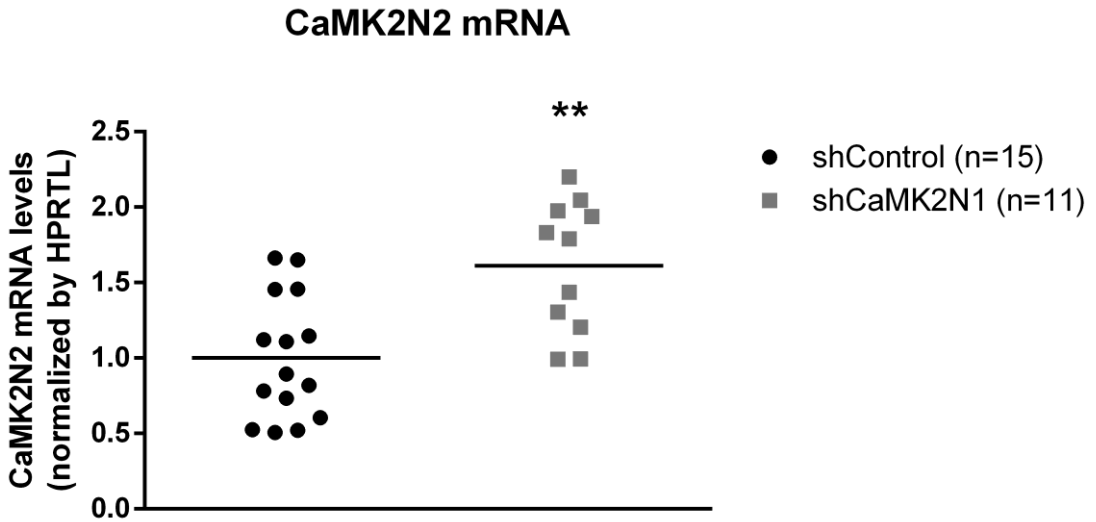

Figure 3.3-4 CaMK2N2 mRNA levels from experiment with CaMK2N1 knockdown
Quantitative analyses of the levels of CaMK2N2 mRNA. CaMK2N2 mRNA levels were normalized by HPRTL mRNA levels. All results were standardize to shControl group average. ** P<0.01

3.4 Effect of CaMK2N1 knockdown on memory maintenance is retrieval dependent

Considering the behavioural phenotype observed (Figure 3.3-2) the memory impairment could be dependent on two variables, they being time and re-exposure. The first memory test is closer in time to the learning session but also animals have not been exposed to a previous memory test when executing the first memory test. To test these two possibilities we subjected animals to only one memory test 4 days after learning session, as can be seen in Figure 3.4-1.

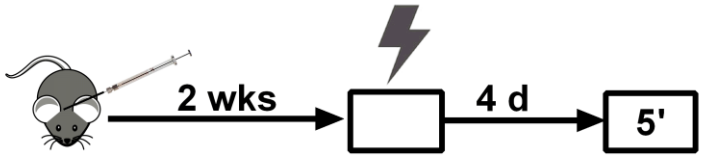

Figure 3.4-1 Experimental design one memory test
The figure shows the experimental design for testing time or re-exposure dependence of the CaMK2N1 knockdown induced memory impairment. Animals were first injected with shControl or shCaMK2N1 virus solution. Two weeks later they were trained with one shock of 0.7 mA. Animals were then re-exposed to the same context for 5 minutes. The test took place 4 days after training session. Animals were sacrificed two hours after the memory test.

If the memory impairment is time-dependent and retrieval-independent, then, 4 days after training the animals should have a memory impairment. However, if the memory impairment is dependent solely on re-exposure to the context we should not observe a memory impairment when the animals are tested only once. As shown in Figure 3.4-2, there was no significant difference between shControl and shCaMK2N1 group if the animals were tested only once ($F_{1,38}$=1.0; P=0.319). This indicates that the memory impairment observed is dependent of re-exposure to the context.

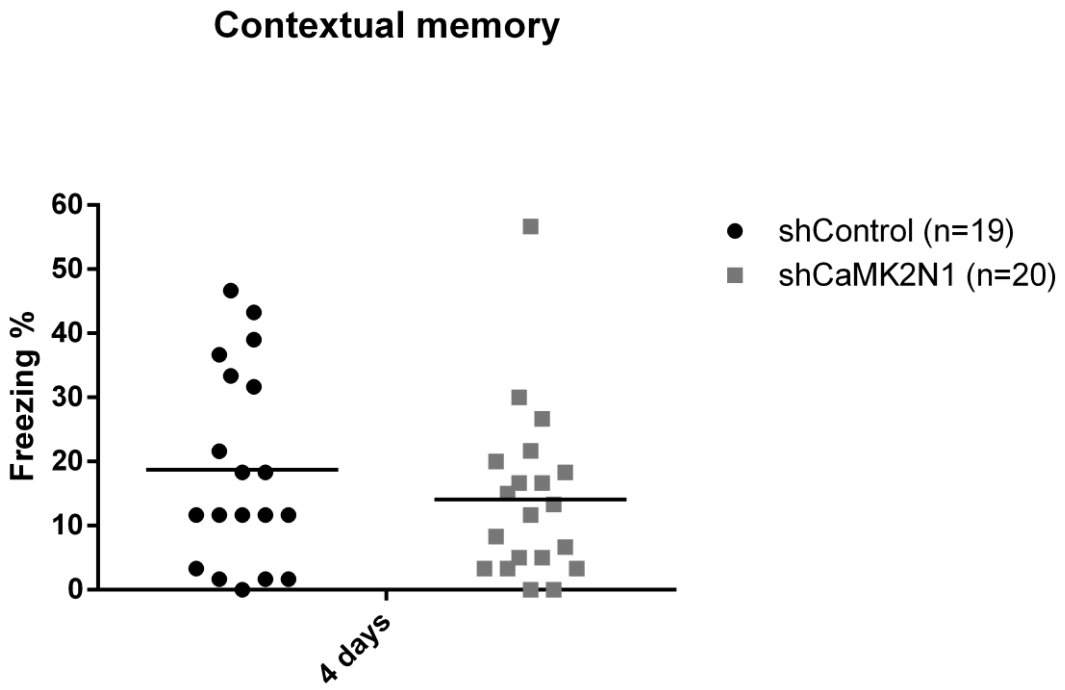

Figure 3.4-2 Freezing scores from experiment with one memory test
This figure shows the average of freezing scores of animals from shControl and shCaMK2N1 groups in one memory test executed 4 days after training. Freezing scores are presented in the ordinate axis as percentage of freezing time in the 5 minutes of test.

RT-qPCR analyses confirmed the efficiency of gene knockdown with a significant decrease of CaMK2N1 mRNA levels in animals treated with shCaMK2N1 virus solution ($F_{1,38}$=66.1; P<0.001) (Figure 3.4-3) and no significant change in CaMK2N2 mRNA levels ($F_{1,38}$=0.08; P=0.769) (Figure 3.4-4).

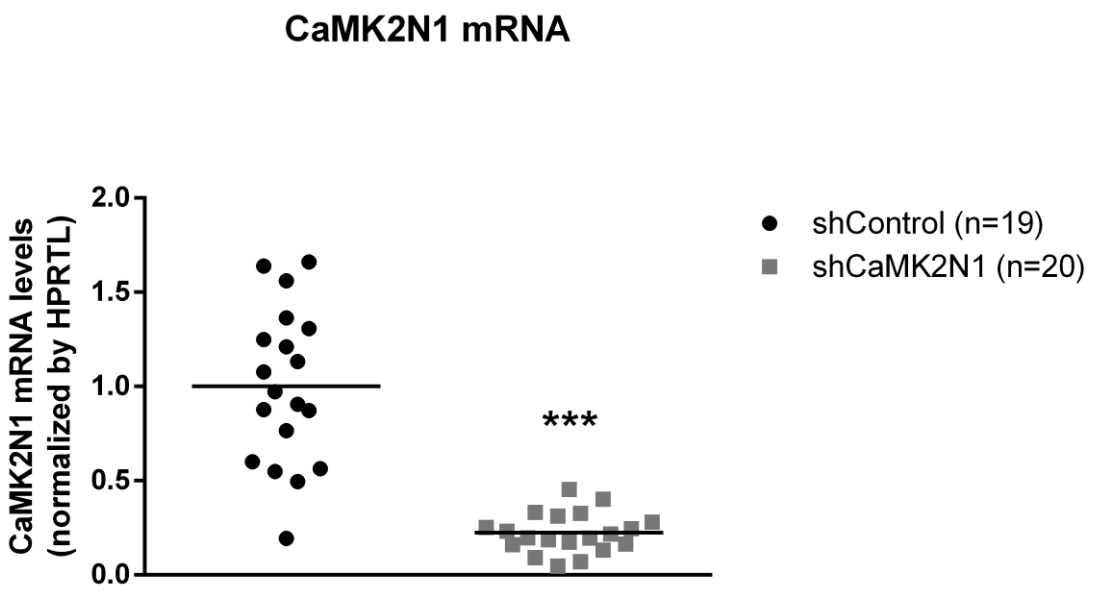

Figure 3.4-3 CaMK2N1 mRNA levels from experiment with one memory test
Quantitative analyses of the levels of CaMK2N1 mRNA. CaMK2N1 mRNA levels were normalized by HPRTL mRNA levels. All results were standardize to shControl group average. *** P<0.001

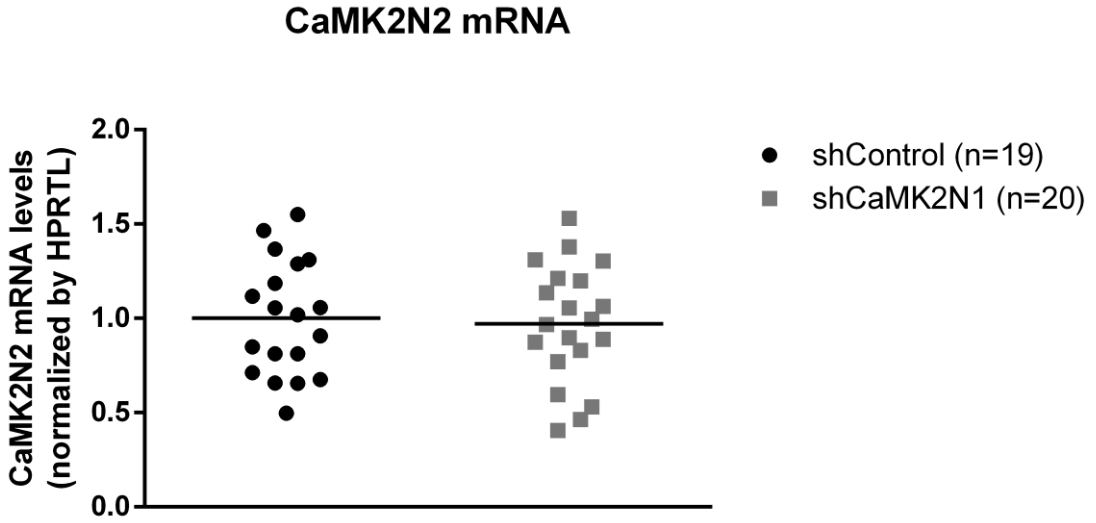

Figure 3.4-4 CaMK2N2 mRNA levels from experiment with one memory test
Quantitative analyses of the levels of CaMK2N2 mRNA. CaMK2N2 mRNA levels were normalized by HPRTL mRNA levels. All results were standardize to shControl group average.

3.5 Retrieval-induced expression of CaMKII inhibitors

To further investigate the relevance of CaMK2N1 and CaMKIIN2 after retrieval of a memory we applied the experimental design showed in Figure 3.5-1 to naïve mice. The aim of this experiment was to look for any retrieval induced change in the expression of the inhibitors of CaMKII. Briefly, animals were placed in the conditioning chamber and received 3 mild foot shocks. One day later animals were either re-exposed to the conditioning chamber for 5 minutes and contextual fear memory tested (TR+TE), or just been trained (TR) in the CFC paradigm.

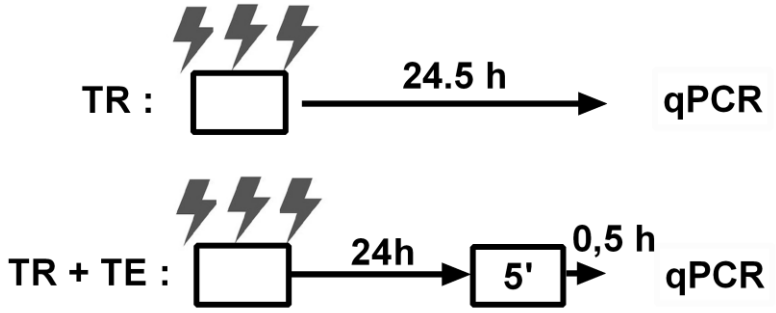

Figure 3.5-1 Experimental design retrieval induced changes on CaMKII inhibitors
The image above presents the experimental design for testing the effects of re-exposure in the expression levels of CaMK2N1 and CaMK2N2. Naïve animals were trained with three shocks of 0.7 mA, for 2 seconds each. Animals from TR+TE group were then re-exposed to the context for one memory tests of 5 minutes. The test took place 24 hours after training session. All animals were sacrificed 30 minutes after the memory test of group TR+TE.

This experiment was previously executed by Dr. Keiko Mizuno, hence the differences in number of shocks and time point of the sacrifice. Dorsal hippocampal samples collected by Dr. Mizuno were used in this thesis to test by RT-qPCR the mRNA levels of CaMK2N1 and CaMK2N2. As shown in Figure 3.5-2, animals subjected to a memory test of 5 minutes (TR+TE) presented an increase in CaMK2N1 mRNA levels when compared to animals without memory test (TR) ($F_{1,9}$=21.8; P=0.002). No significant change in CaMK2N2 mRNA levels was observed ($F_{1,9}$=0.04; P=0.842) (Figure 3.5-3).

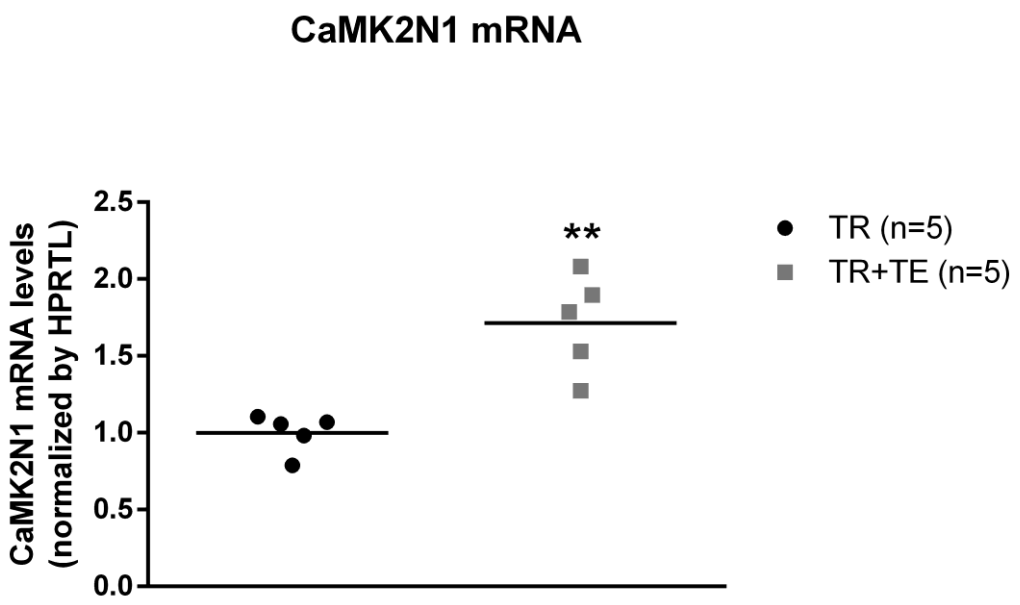

Figure 3.5-2 CaMK2N1 mRNA levels after memory retrieval
Quantitative analyses of the levels of CaMK2N1 mRNA with or without a memory test. Animals from group TR were just trained with the CFC paradigm whilst animals from group TR+TE were trained and tested with the CFC paradigm. CaMK2N1 mRNA levels were normalized by HPRTL mRNA levels. All results were standardize to shControl group average. ** P<0.01

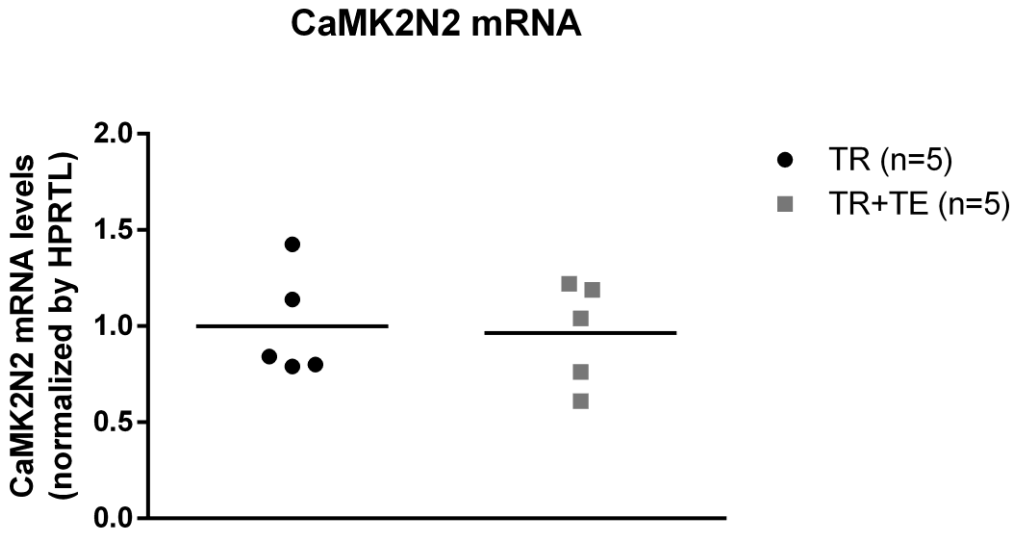

Figure 3.5-3 CaMK2N2 mRNA levels after memory retrieval
Levels of CaMK2N2 mRNA with or without a memory test are presented in the graph. Animals from group TR were just trained with the CFC paradigm whilst animals from group TR+TE were trained and tested with the CFC paradigm. CaMK2N2 mRNA levels were normalized by HPRTL mRNA levels. All results were standardize to shControl group average.

On the other hand, RT-qPCR analyses of samples from a CFC experiment with 5 shocks, also previously executed by Dr. Keiko Mizuno, revealed a different regulation of the inhibitors. Experimental design for this five shocks experiment can be seen in Figure 3.5-4. It is the same experimental design as for training with three shocks.

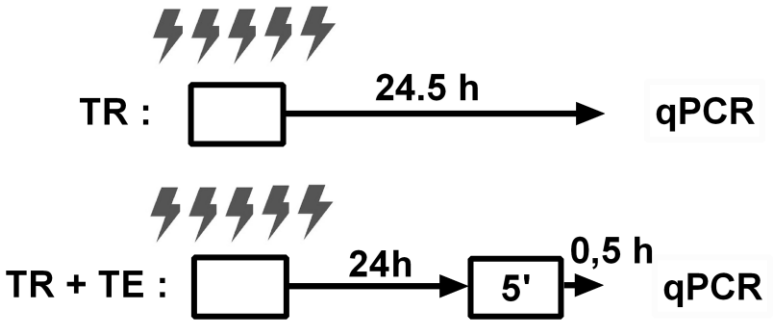

Figure 3.5-4 Experimental design retrieval with 5 shocks learning session
As the experimental design in Figure 3.5-1 the scheme above shows an experimental design to test the effect of the memory test but after a 5 shocks learning session. Naïve animals were trained with five shocks of 0.7 mA, for 2 seconds each. Animals from TR+TE group were then re-exposed to the context for one memory tests of 5 minutes. The test took place 24 hours after training session. All animals were sacrificed 30 minutes after the memory test of group TR+TE.

RT-qPCR analyses shows no significant difference between the groups on the levels of CaMK2N1 mRNA ($F_{1,11}$=1.8; P=0.206) (Figure 3.5-5). However, a significant increase in the mRNA levels for CaMK2N2 was observed in animals from group TR+TE ($F_{1,11}$=5.4; P=0.041) (Figure 3.5-6). This difference on the effect of memory retrieval in the regulation of the inhibitors dependent of the intensity of the US can be interpreted as indication of different functions for each inhibitor. This will be discussed further in Chapter 5.

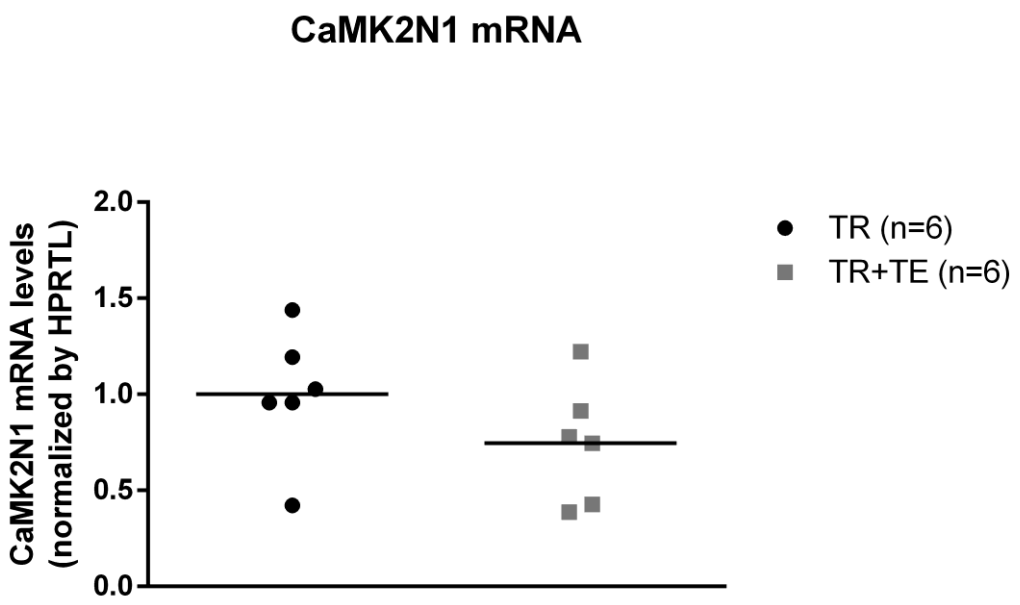

Figure 3.5-5 CaMK2N1 mRNA levels after 5 shocks memory retrieval
Quantitative analyses of the levels of CaMK2N1 mRNA with or without a memory test, after a 5 shocks memory test. Animals from group TR were just trained with a 5 shocks learning session, whilst animals from group TR+TE were trained and tested with in the CFC paradigm. CaMK2N1 mRNA levels were normalizes by HPRTL mRNA levels. All results were standardize to shControl group average.

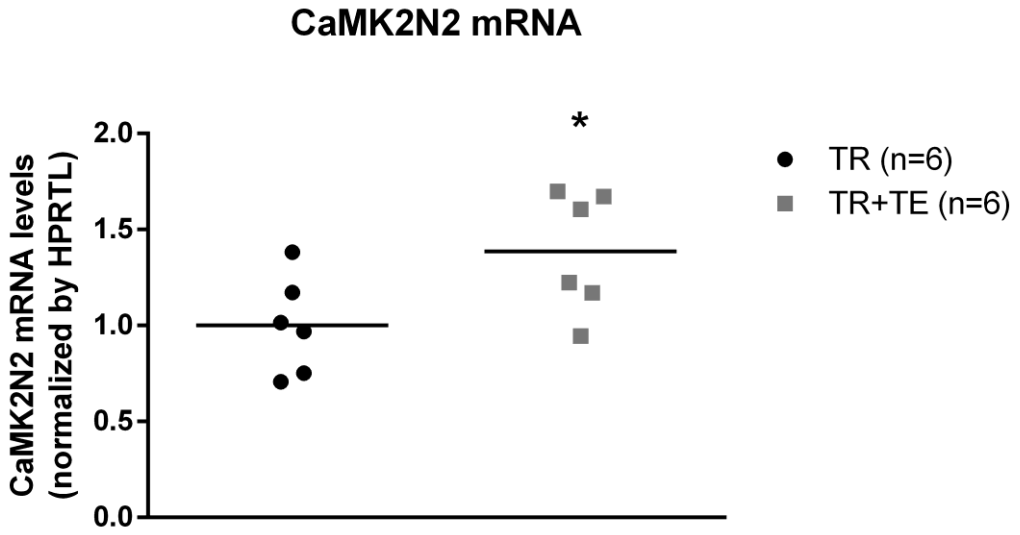

Figure 3.5-6 CaMK2N2 mRNA levels after 5 shocks memory retrieval
CaMK2N2 mRNA levels are presented in the graph above. Animals from group TR were just trained with 5 shocks learning session the CFC paradigm. Animals from group TR+TE were trained and tested. CaMK2N2 mRNA levels were normalizes by HPRTL mRNA levels. All results were standardize to shControl group average. *P<0.05

3.6 Molecular effects of CaMK2N1 knockdown

To better understand the molecular changes caused by CaMK2N1 knockdown we analysed the expression and phospho-regulation of different proteins by western blot. The analysis consisted of 4 groups of experiments. The first group of western blots aimed to study the effect of shCaMK2N1 treatment on CaMKII signalling. The second group of experiments looked at the effect of CaMK2N1 knockdown on expression of the AMPA receptor subunit GluA1. The third group of western blots studied changes in protein degradation. The last group of studies investigated changes in molecules involved in regulation of gene expression.

3.6.1 Effects of CaMK2N1 knockdown on CaMKII

Mice were either injected with shControl or shCaMK2N1 virus solution. Two weeks later animals were subjected to the CFC paradigm, as presented in Figure 3.6-1. The animals were divided between does just trained (TR) and does trained and tested (TR+TE) in the CFC paradigm. Two hours after the end of the memory test session their dorsal hippocampus was collected for western blot analyses.

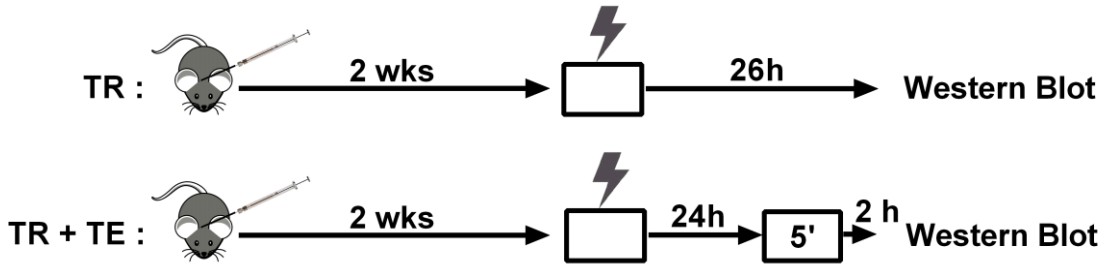

Figure 3.6-1 Experimental design for western blot analyses
The image above presents the experimental design applied to the animals used for western blot analyses. Naïve animals were trained with one shocks of 0.7 mA, for 2 seconds each. Animals from TR+TE group were then re-exposed to the context for one memory tests of 5 minutes whilst animals from TR group were not tested. The test took place 24 hours after training session. All animals were sacrificed 2 hours after the memory test of group TR+TE.

Western blot analyses showed no significant change in total αCaMKII expression in crude synaptosomal fraction (P2) when normalized to synaptotagmin, as shown of Figure 3.6-2. Two-way ANOVA found no significant effect of memory test ($F_{1,27}$=0.02; P=0.889), CaMK2N1 knockdown ($F_{1,27}$=0.4; P=0.488) or interaction ($F_{1,27}$=0.1; P=0.748). However, analyses of P2 fraction showed a significant difference in levels of T286-phosphorylated αCaMKII (Figure 3.6-3). Two-way ANOVA test showed a significant effect of CaMK2N1 knockdown ($F_{1,26}$=6.6; P=0.017). No significant effect for memory test ($F_{1,26}$=3.8; P=0.064) or interaction between the memory test and CaMK2N1 knockdown ($F_{1,26}$=1.8; P=0.185) was observed. SNK planned comparisons showed a significant decrease in phosphorylated αCaMKII after memory test within the shControl group (q=3.1; P=0.035) but not shCaMK2N1 group (q=0.6; P=0.670). A significant difference in αCaMKII autophosphorylation level was also observed between shControl and shCaMK2N1 after memory retrieval (q=3.7; P=0.014) but not without it (q=1.2; P=0.382).

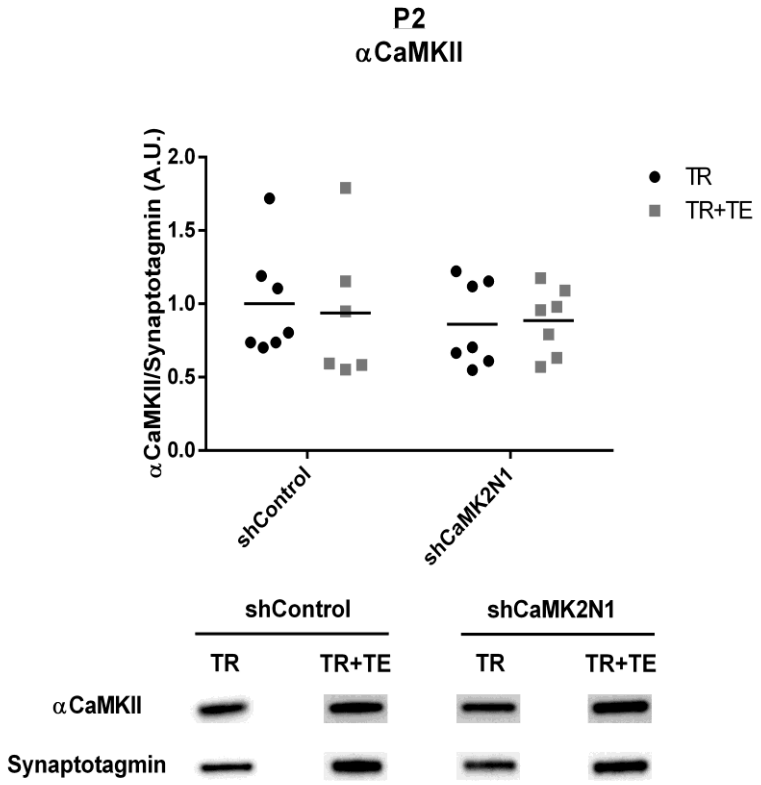

Figure 3.6-2 P2 αCaMKII levels after CaMK2N1 knockdown
This figure shows the levels of αCaMKII for animals trained (TR) or trained and tested (TR+TE) in the CFC paradigm. Animals of both groups also received intra-hippocampal injection of shControl or shCaMK2N1 viral solution. Levels of αCaMKII were normalized by the internal loading control, Synaptotagmin. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show example bands of αCaMKII and Synaptotagmin for all groups.

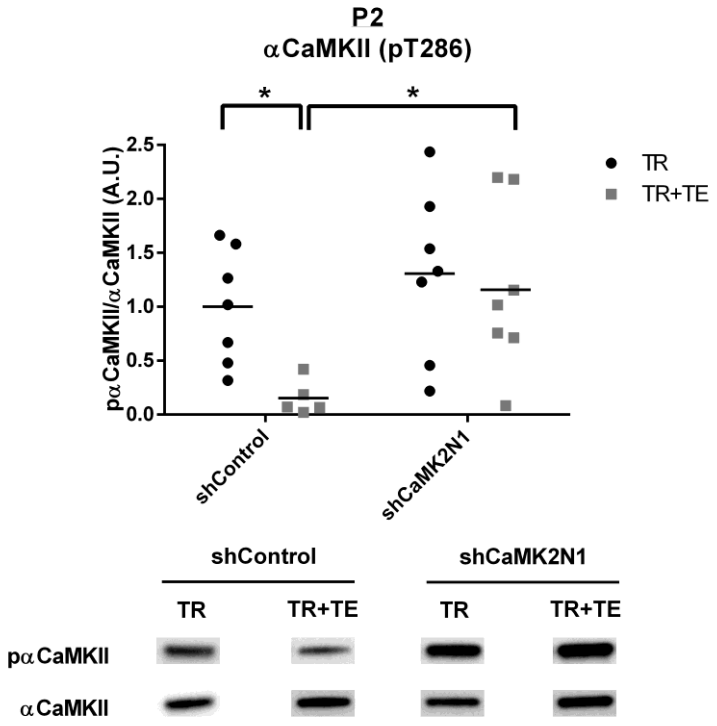

Figure 3.6-3 P2 T286 phosphorylated αCaMKII levels after CaMK2N1 knockdown
This figure shows the levels of T286 phosphorylated αCaMKII (pαCaMKII) for animals trained (TR) or trained and tested (TR+TE) in the CFC paradigm. Animals of both groups also received intra-hippocampal injection of shControl or shCaMK2N1 viral solution. Levels of phosphorylated αCaMKII were normalized by total αCaMKII levels. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show example bands of pαCaMKII and αCaMKII for all groups. *P<0.05

We also analysed the levels of total (Figure 3.6-4) and T286 autophosphorylated (Figure 3.6-5) αCaMKII in cytosolic fraction (S2). There was no significant effect of memory test ($F_{1,26}$=1.1; P=0.300), virus treatment ($F_{1,26}$=2.2; P=0.145) or interaction effect ($F_{1,26}$=3.3; P=0.079) in total αCaMKII levels. We also observed no effect of memory test ($F_{1,24}$=0.7; P=0.389), CaMK2N1 knockdown ($F_{1,24}$=4.0; P=0.058) or interaction between factors ($F_{1,24}$=0.4; P=0.512) when measuring phosphorylated αCaMKII levels in S2 fraction. As can be deduced by the results above presented, although some tendency effects in αCaMKII levels and phosphorylation were seen in the S2 samples, the effects of memory retrieval and CaMK2N1 were more pronounced in the synaptosomal fraction where they reached significant levels.

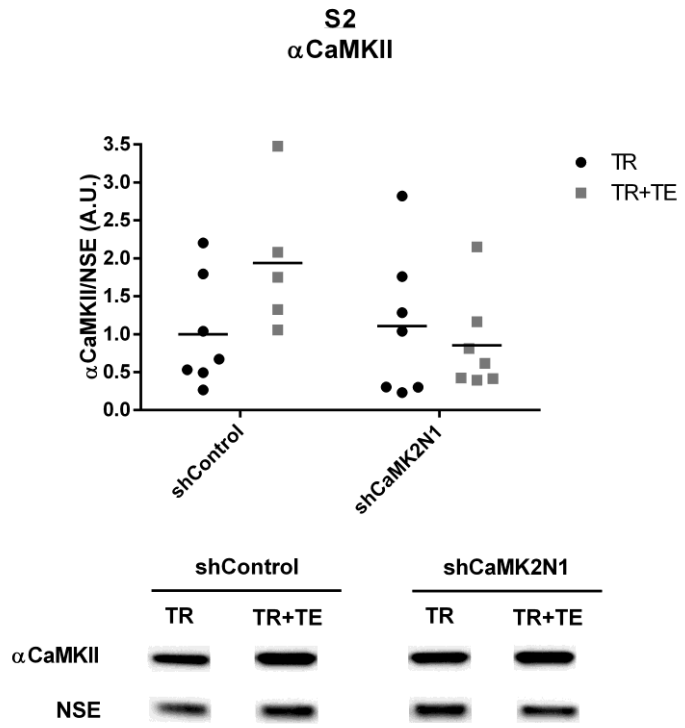

Figure 3.6-4 S2 αCaMKII levels after CaMK2N1 knockdown
This figure shows the levels of αCaMKII in the S2 fraction of animals trained (TR) or trained and tested (TR+TE) in the CFC paradigm. Animals of both groups also received intra-hippocampal injection of shControl or shCaMK2N1 viral solution. Levels of αCaMKII were normalized by the internal loading control, Synaptotagmin. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show exemple bands of αCaMKII and Synaptotagmin for all groups.

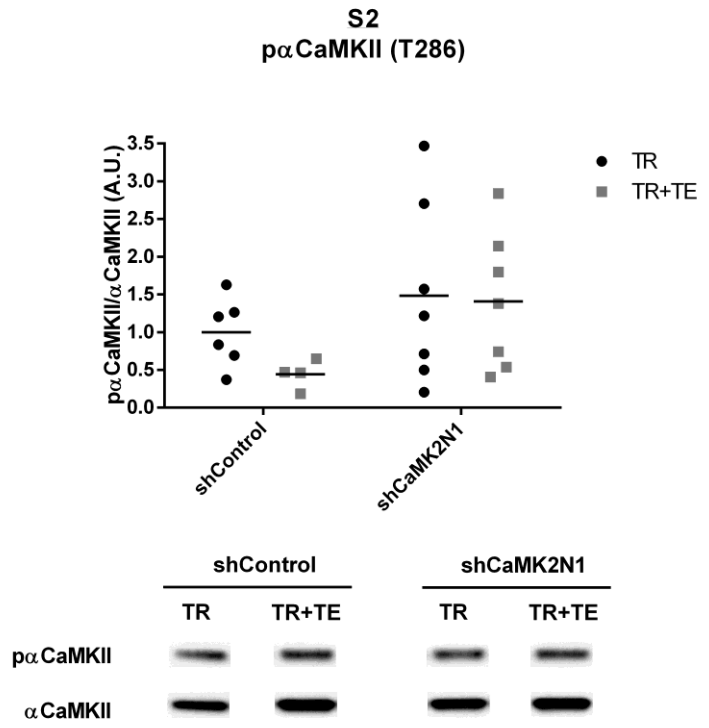

Figure 3.6-5 S2 T286 phosphorylated αCaMKII levels after CaMK2N1 knockdown
This figure shows the levels of T286 phosphorylated αCaMKII (pαCaMKII) in the S2 fraction of animals trained (TR) or trained and tested (TR+TE) in the CFC paradigm. Animals of both groups also received intra-hippocampal injection of shControl or shCaMK2N1 viral solution. Levels of phosphorylated αCaMKII were normalized by total αCaMKII levels. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show example bands of pαCaMKII and αCaMKII for all groups.

3.6.2 Effects of CaMK2N1 knockdown on GluA1

It is known that the AMPAR subunit GluA1 is a phosphorylation target of CaMKII (Derkach et al., 1999), and that CaMKII can regulate the distribution of AMPAR at the synapse (Opazo et al., 2010). Both processes of AMPAR regulation by CaMKII have been suggested to be important for memory formation and maintenance (Lisman et al., 2002; Lisman et al., 2012; Sanhueza and Lisman, 2013). Therefore, it was relevant to study the effect of CaMK2N1 knockdown on total (Figure 3.6-6) and S831-phosphorylated (Figure 3.6-7) levels of GluA1. The same samples which were used for the CaMKII western blot studies were probed to invesitgate GluA1 expression and regulation (Figure 3.6-1). Western blot results indicated a significant effect of CaMK2N1 knockdown in total GluA1 levels in P2 fraction ($F_{1,25}$=4.9; P=0.038). No effect of the memory test ($F_{1,25}$=0.5;

P=0.468) and no interaction between both factors ($F_{1,25}$=1.3; P=0.261) was observed. SNK planned comparisons showed a significant difference in GluA1 levels between shControl and shCaMK2N1 animals only when animals were trained and tested (TR+TE) (q=3.2; P=0.030), but not when they were only trained (TR) (q=1.0; P=0.448). No significant effect of memory test within shControl (q=1.7; P=0.220) or shCaMK2N1 (q=0.4; P=0.757) groups was observed (Figure 3.6-6).

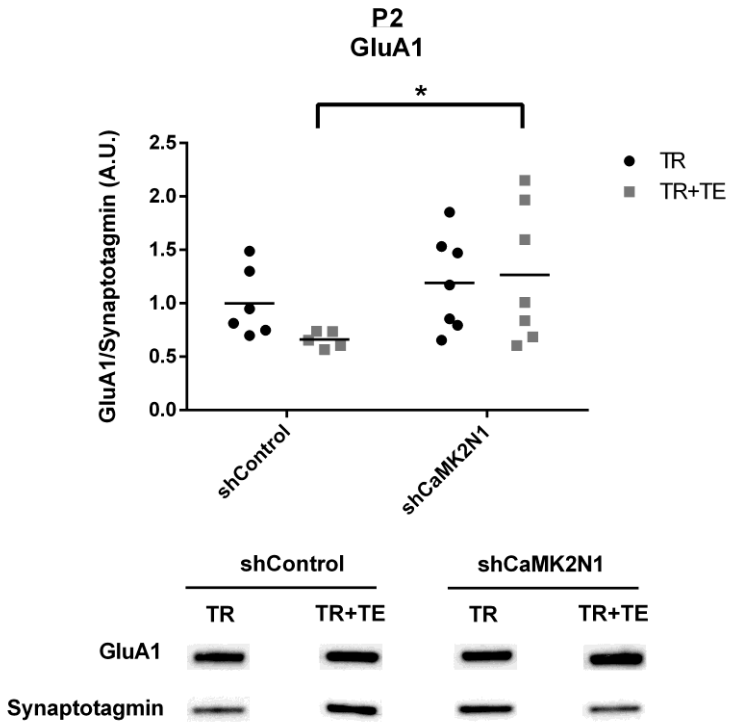

Figure 3.6-6 P2 GluA1 levels after CaMK2N1 knockdown
This figure shows the levels of GluA1 in the P2 fraction of animals injected with shControl or shCaMK2N1 virus solution (as indicated on coordinate axe). Animals were also divided between those subjected to only training at CFC paradigm (TR) and those trained and tested for memory of context (TR+TE). Levels of GluA1 were normalized by the internal loading control, Synaptotagmin. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show exemple bands of GluA1 and Synaptotagmin for all groups. *P<0.05

P2 levels of phosphorylated GluA1 presented a significant decrease as effect of ShCaMK2N1 virus treatment ($F_{1,25}$=6.9; P=0.015) (Figure 3.6-7). Once more, SNK planned comparisons shows this decrease to be significant only in TR+TE animals (q=3.0; P=0.046) but not in TR animals (q=2.2; P=0.124). Two-way ANOVA also showed no effect of memory test ($F_{1,25}$= 1.6; P=0.207) or interaction ($F_{1,25}$=0.1; P=0.668). No significant

difference between TR and TR+TE animals was observed within the shControl animals (q=1.6; P=0.260) or within shCaM2N1 (q=0.9; P=0.520) animals, by SNK planned comparisons.

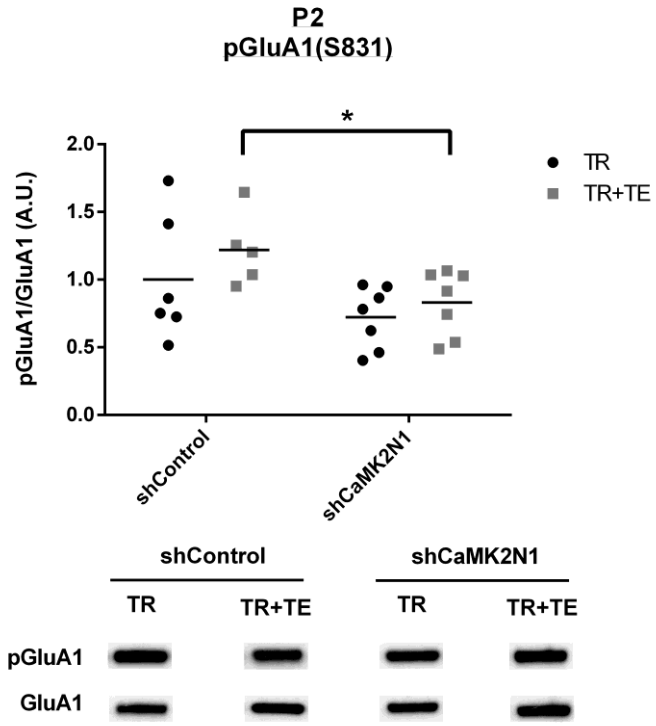

Figure 3.6-7 P2 Phosphorylated GluA1 levels after CaMK2N1 knockdown
The figure above shows the levels of S831 phosphorylated GluA1 in the P2 fraction of animals injected with shControl or shCaMK2N1 virus solution (as indicated on coordinate axe). Animals were also divided between those subjected to only training at CFC paradigm (TR) and those trained and tested for memory of context (TR+TE). Levels of pGluA1 were normalized by total GluA1 levels. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show exemple bands of pGluA1 and GluA1 for all groups. *P<0.05

The fact that the effects of CaMK2N1 knockdown on total and phosphorylated GluA1 levels were only significant in TR+TE animals seems to indicate that these effects are dependent on memory retrieval. However, since both groups, TR and TR+TE, were subjected to CFC training it was not possible to investigate any baseline effect of shCaM2N1 virus treatment on GluA1 levels and phosphorylation. To test for any GluA1 alterations induced by CaMK2N1 knockdown in naïve animal we tested the effect of CaMK2N1 knockdown on naïve animals, as presented on Figure 3.6-8.

Figure 3.6-8 Experimental design for western blot of naive animals
The image above presents the experimental design applied to study the effects of CaMK2N1 knockdown in naïve animals. Naïve animals received an intra-hipocampal injection of either shControl virus solution of shCaMK2N1 virus solution. All animals were sacrificed 15 days after injection.

Treatment with shCaMK2N1 virus solution in naïve animals had no effect on total GluA1 ($F_{1,16}$=2.1; P=0.161) (Figure 3.6-9) or pGluA1 ($F_{1,17}$=0.6; P=0.443) (Figure 3.6-10) levels. This indicated that the GluA1 alterations induced by CaMK2N1 knockdown are not due to baseline alterations but rather a learning/memory dependent effect.

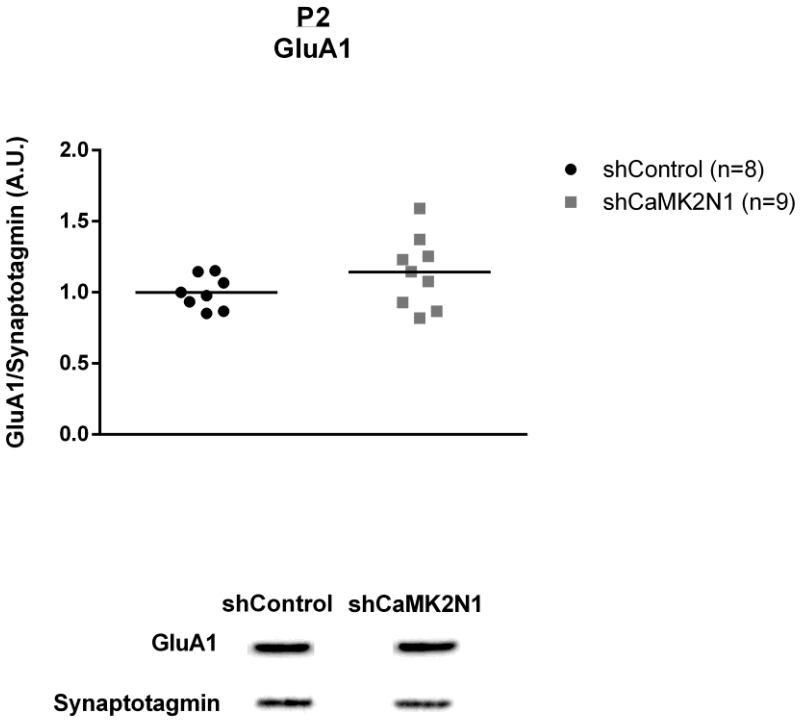

Figure 3.6-9 P2 GluA1 levels after CaMK2N1 knockdown in naive animals
This figure shows the levels of GluA1 in the P2 fraction of naive animals injected with shControl or shCaMK2N1 virus solution. Levels of GluA1 were normalized by the internal loading control, Synaptotagmin. The results were standardized to shControl average. Images on the bottom of the figure show exemple bands of GluA1 and Synaptotagmin for all groups.

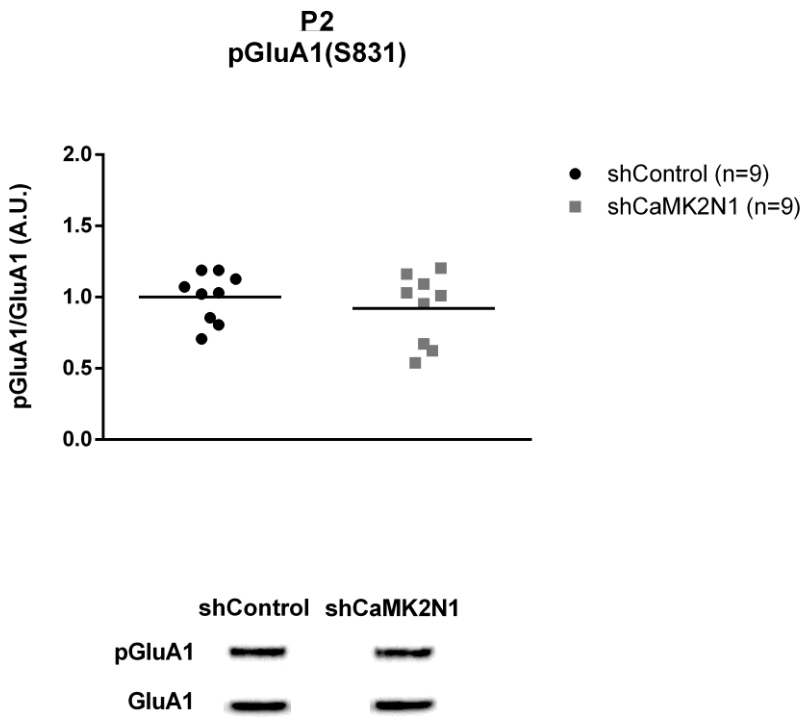

Figure 3.6-10 P2 pGluA1 levels after CaMK2N1 knockdown in naive animals
The figure above shows the levels of S831 phosphorylated GluA1 in the P2 fraction of naive animals injected with shControl or shCaMK2N1 virus solutiont. Levels of pGluA1 were normalized by total GluA1 levels. The results were standardized to shControl average. Images on the bottom of the figure show exemple bands of pGluA1 and GluA1 for all groups.

3.6.3 Effects of CaMK2N1 knockdown on protein degradation

Protein degradation by the proteasome system has been shown to be an important process for memory consolidation (Jarome et al., 2011; Lopez-Salon et al., 2001) and reconsolidation (Artinian et al., 2008; Lee et al., 2008). Additionally, CaMKII has been show to regulate proteasome activity by phosphorylation of the Rpt6 subunit (Djakovic et al., 2009; Jarome et al., 2011) and proteasome localization in the PSD area of the synapse by acting as a scaffold protein (Bingol et al., 2010). These memory related processes of protein degradation have a few known targets like GluA1 (Naskar et al., 2014) and the scaffolding protein Shank (Lee et al., 2008). In the case of Shank, Lee et al. (2008) have observed an increase in Shank degradation 2 hours after CFC memory retrieval session. Due to the similarity of the experimental design used by Lee et al. (2008) and the one used in our experiments, we decided to measure Shank's levels in

the P2 fraction as an indirect measure of protein degradation at the synapses. For that propose P2 fraction of the dorsal hippocampus of animals subjected to the experimental design described in Figure 3.6-1 were used for western blot analysis. Three bands, probably corresponding to the three known isoforms of Shank, were detected and analysed together. The effect of viral treatment, memory retrieval session and interaction between these two factors were tested by two-way ANOVA. No significant effect of virus treatment ($F_{1,27}$=0.6; P=0.419), memory test ($F_{1,27}$=0.05; P=0.811), or interaction between both factors ($F_{1,27}$=0.7; P=0.393) were observed (Figure 3.6-11). Analyses of each one of the three bands separately also showed no significant result [Top band: virus effect ($F_{1,27}$=0.3; P=0.580), memory effect ($F_{1,27}$=0.2; P=0.652), interaction effect ($F_{1,27}$=0.7; P=0.382); Middle band: virus effect ($F_{1,27}$=0.2; P=0.616), memory effect ($F_{1,27}$=0.3; P=0.573), interaction effect ($F_{1,27}$=0.5; P=0.479); Lower band: virus effect ($F_{1,27}$=1.4; P=0.238), memory effect ($F_{1,27}$<0.001; P=0.983), interaction effect ($F_{1,27}$=0.4; P=0.515)].

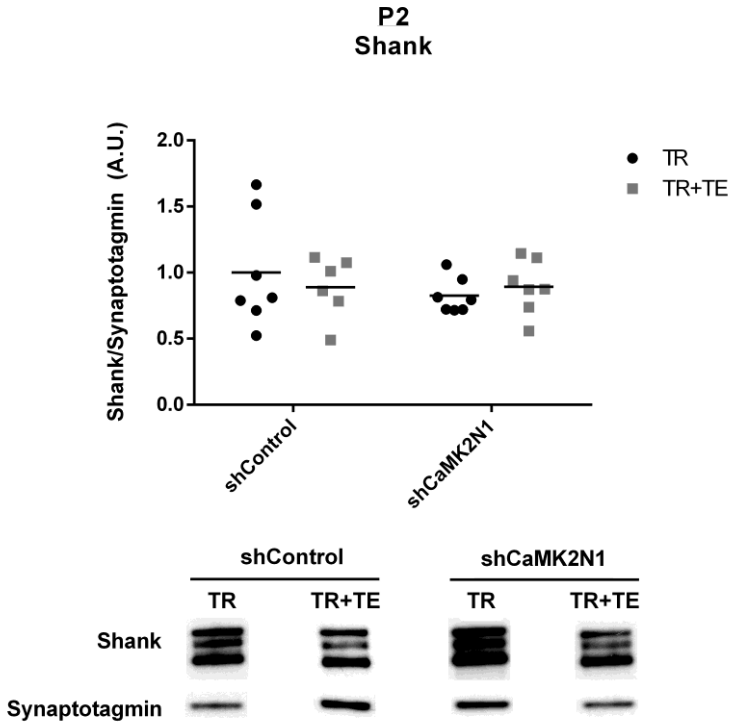

Figure 3.6-11 P2 Shank levels after CaMK2N1 knockdown
The figure above shows the levels of Shank protein in the P2 fraction of animals injected with shControl or shCaMK2N1 virus solution (as indicated on coordinate axe). Animals were also divided between those subjected to only training at CFC paradigm (TR) and those trained and tested for memory of context (TR+TE). Levels of Shank were normalized by Synaptotagmin levels. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show example bands of Shank and Synaptotagmin for all groups.

Proteasome degradation of proteins is regulated by ubiquitination of target proteins (Ciechanover and Stanhill, 2014). In the same article that Lee et al. (2008) reported the memory retrieval induced degradation of Shank an increase in ubiquitination of synaptosomal proteins was also reported. Increased ubiquitination of proteins following memory retrieval has also been shown to occur by Fukushima et al. (2014). Therefore, we decided to quantify polyubiquitination levels at the P2 fraction of our samples. Two-way ANOVA test showed no significant effect of memory retrieval ($F_{1,27}$=0.1; P=0.747), CaMK2N1 knockdown ($F_{1,27}$=0.4; P=0.530) or interaction ($F_{1,27}$=0.1; P=0.701) in the levels of P2 ubiquitinated proteins (Figure 3.6-12).

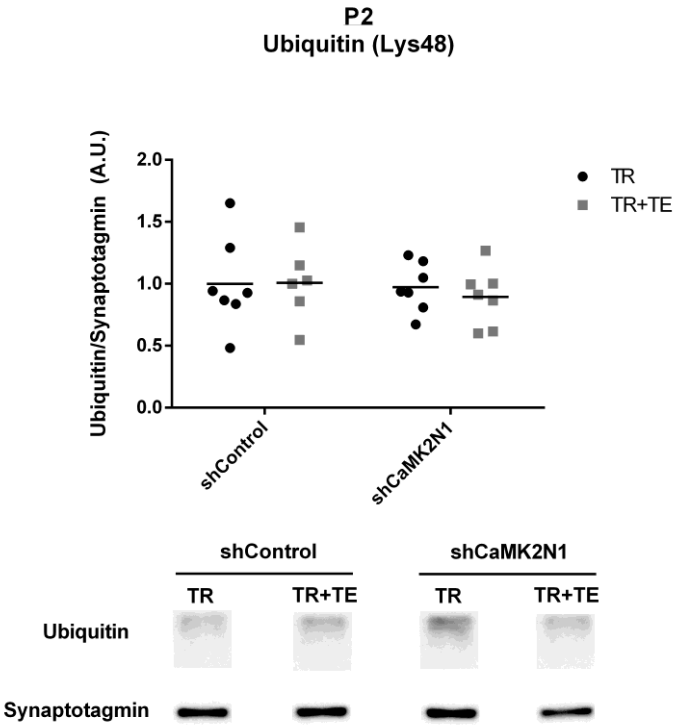

Figure 3.6-12 P2 Ubiquitinated proteins levels after CaMK2N1 knockdown
The image shows the levels of ubiquitinated proteins in the P2 fraction of animals injected with shControl or shCaMK2N1 virus solution. Animals were also divided between those subjected to only training at CFC paradigm (TR) and those trained and tested for memory of context (TR+TE). Levels of ubiquitineted proteins were normalized by Synaptotagmin levels. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show example bands of ubiquitinated proteins (seen as a smear band due to the various size of proteins been ubiquitinated) and Synaptotagmin for all groups.

Finally, we measured the levels of phosphorylated (Figure 3.6-14) and total (Figure 3.6-13) proteasome subunit Rpt6. There was no significant effect of shCaMK2N1 treatment ($F_{1,26}$<0.001; P=0.987), memory retrieval ($F_{1,26}$=0.2; P=0.639) or interaction ($F_{1,26}$=0.02; P=0.876) in the levels of total Rpt6 (Figure 3.6-13). Two-way ANOVA test also showed no effect of virus treatment ($F_{1,22}$=0.3; P=0.543), memory test ($F_{1,22}$=0.8; P=0.383) or interaction between these two factors ($F_{1,22}$=0.6; P=0.424) in the levels of Rpt6 phosphorylation (Figure 3.6-14).

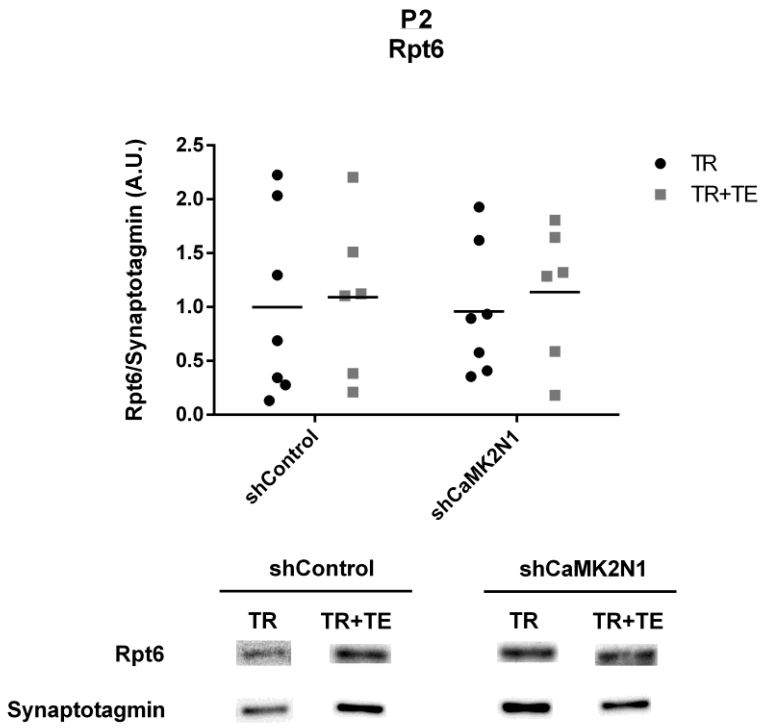

Figure 3.6-13 P2 Rpt6 levels after CaMK2N1 knockdown

The figure above shows the levels of the proteasome sub-unit Rpt6 in the P2 fraction of animals injected with shControl or shCaMK2N1 virus solution (as indicated on coordinate axe). Animals were also divided between those subjected to only training at CFC paradigm (TR) and those trained and tested for memory of context (TR+TE). Levels of Rpt6 were normalized by Synaptotagmin levels. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show example bands of Rpt6 and Synaptotagmin for all groups.

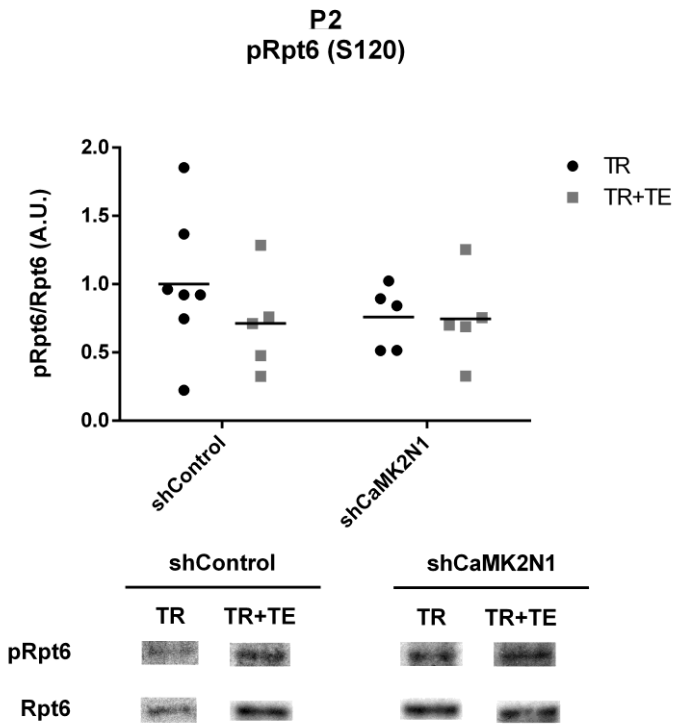

Figure 3.6-14 P2 phosphorylated Rpt6 levels after CaMK2N1 knockdown
This image shows the levels of phosphorylated Rpt6 in the P2 fraction of animals injected with shControl or shCaMK2N1 virus solution. Animals were also divided between those subjected to only training at CFC paradigm (TR) and those trained and tested for memory of context (TR+TE). Levels of pRpt6 were normalized by total Rpt6 levels. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show example bands of pRpt6 and total Rpt6 for all groups.

Taken together, our indirect and direct observations of the proteasome system seems to indicate that the behavioural phenotype observed as a consequence of CaMK2N1 knockdown is not related to protein degradation by the proteasome system.

3.6.4 Effects of CaMK2N1 knockdown on gene expression

Gene expression and protein synthesis have been shown to be necessary for memory maintenance after a retrieval session(Kelly et al., 2003; Lee et al., 2004). Gene expression is known to be regulated by CaMKII kinase activity via different pathways (Chen et al., 2012; Takeuchi et al., 2002; Wang et al., 2013), hence gene expression changes are a good candidate for a molecular basis of the behavioural phenotype observed in our experiments after CaMK2N1 knockdown. To study the effects of

CaMK2N1 knock down in retrieval-induced gene expression we used an earlier time point after retrieval session, of 1 hour after, and extracted P1 fraction of the samples. In order to do so, the experimental design explained in Figure 3.6-15 was applied. It is basically the same experimental design used previously for other western blot analysis with the difference that animals from group TR+TE were sacrificed 1 hour after memory retrieval session.

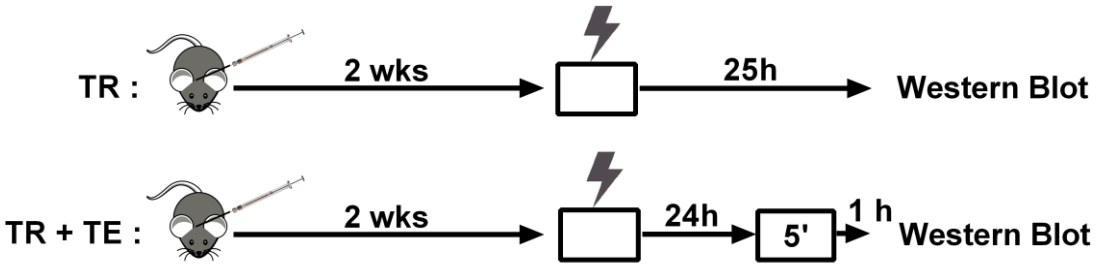

Figure 3.6-15 Experimental design for western blot analyses of P1 fraction
The image above presents the experimental design applied to the animals used for western blot analyses of P1 fraction. Naïve animals were trained with one shocks of 0.7 mA, for 2 seconds each. Animals from TR+TE group were then re-exposed to the context for one memory tests of 5 minutes whilst animals from TR group were not tested. The test took place 24 hours after training session. All animals were sacrificed 1 hours after the memory test of group TR+TE.

We first analysed the levels of the transcription factor CREB, which is known to be regulated by CaMKII (Ma et al., 2014a). Total levels of CREB (Figure 3.6-16) and its phosphorylation at S133 (Figure 3.6-17) in P1 fraction of all animals were studied by western blot analysis. Two-way ANOVA showed no significant effect of the virus treatment ($F_{1,34}$=1.9; P=0.172), memory test ($F_{1,34}$=1.8; P=0.183) or interaction between both factors ($F_{1,34}$=0.03; P=0.858) in total CREB levels. There was also no significant difference between the groups in terms of CREB S133 phosphorylation (P=0.818), as shown by Kruskal-Wallis test.

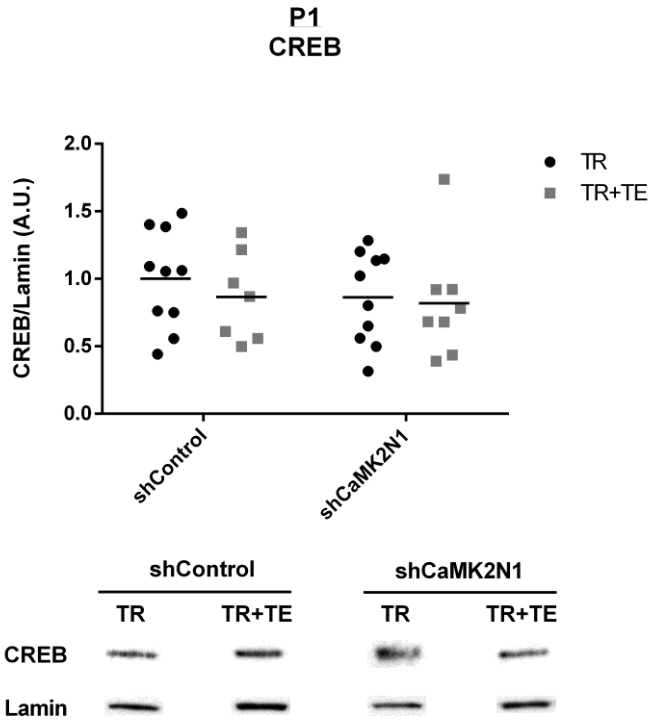

Figure 3.6-16 P1 CREB levels after CaMK2N1 knockdown

The image above shows the levels of CREB in the P1 fraction of animals injected with shControl or shCaMK2N1 virus solution. Animals were also divided between those subjected to only training at CFC paradigm (TR) and those trained and tested for memory of context (TR+TE). Levels of CREB were normalized by nuclear lamin. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show example bands of CREB and Lamin for all groups.

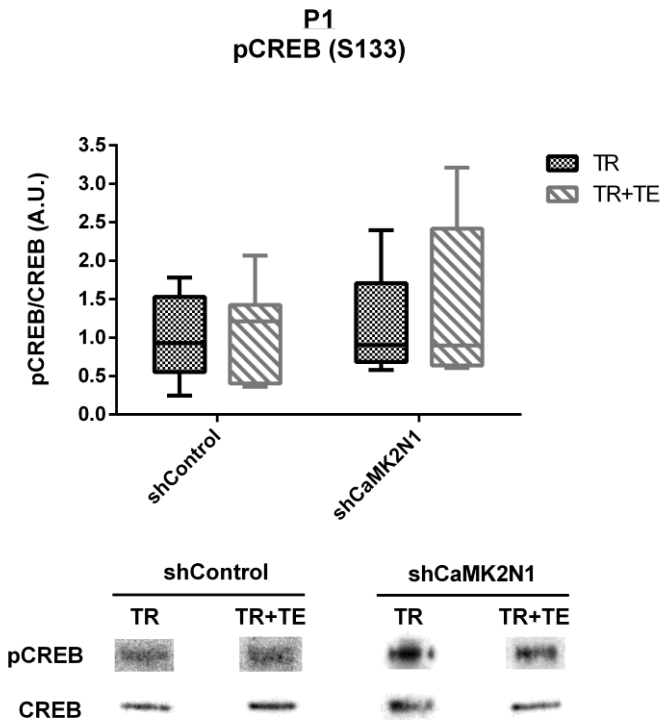

Figure 3.6-17 P1 phosphorylated CREB levels after CaMK2N1 knockdown
This figure shows the levels of phosphorylated CREB in the P1 fraction of animals injected with shControl or shCaMK2N1 virus solution. Animals were also divided between those subjected to only training at CFC paradigm (TR) and those trained and tested for memory of context (TR+TE). Levels of pCREB were normalized by total CREB levels. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show example bands of pCREB and CREB for all groups. The image presents a box plot graph since the distribution of the data was not normal. Median, 2nd and 3rd quartiles, and maximum and minimum values are presented in the graph.

We then analysed the levels of the immediate early gene Zif268 (Figure 3.6-18), a transcription factor important for memory maintenance after retrieval (Lee, 2008; Lee et al., 2004). Two-way ANOVA of the western blot data from P1 fraction showed no significant effect of CaMK2N1 knockdown ($F_{1,33}$=0.02; P=0.887) or memory retrieval ($F_{1,33}$=0.04; P=0.842). However, there was a significant interaction between virus treatment and memory test ($F_{1,33}$=5.7; P=0.023). Although a significant effect of interaction was observed, SNK planned comparisons did not show any significant difference. There was no significant effect of memory retrieval in shControl group (q=2.2; P=0.126) or in shCaMK2N1 group (q=2.5; P=0.079). There was also no significant effect of CaMK2N1 knockdown between trained animals (q=2.4; P=0.094) or in trained and tested animals (q=2.3; P=0.104).

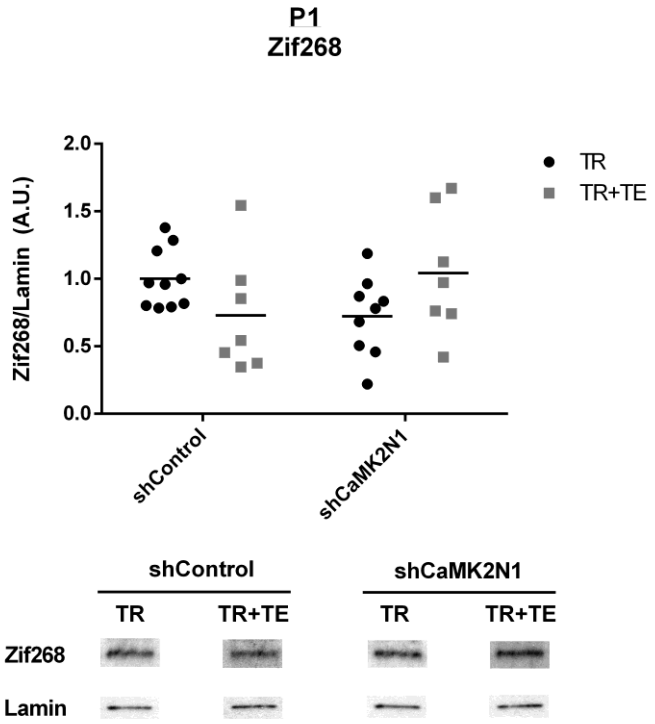

Figure 3.6-18 P1 Zif268 levels after CaMK2N1 knockdown
This figure shows the levels of Zif268 in the P1 fraction of animals injected with shControl or shCaMK2N1 virus solution. Animals were also divided between those subjected to only training at CFC paradigm (TR) and those trained and tested for memory of context (TR+TE). Levels of Zif268 were normalized by Lamin. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show example bands of Zif268 and Lamin for all groups.

Another immediate-early gene analysed was the transcription factor c-fos (Figure 3.6-19). This transcription factor has been show previously to be important for memory consolidation and extinction (Guedea et al., 2011; Huff et al., 2006; Tronson et al., 2009). Again, P1 fraction was extracted from dorsal hippocampus samples and analysed by western blot. Two-way ANOVA revealed no significant effect of virus treatment alone ($F_{1,31}$=0.08; P=0.779) or memory test ($F_{1,31}$=2.7; P=0.110), but there was a significant interaction between these two factors ($F_{1,31}$=10.7; P=0.003). SNK planned comparisons showed a significant effect of memory retrieval within shControl group (q=4.7; P=0.003) but not within shCaMK2N1 group (q=1.7; P=0.239). Additionally, a significant difference between shControl and shCaMK2N1 groups was observed within trained (q=3.4; P=0.022) and trained and tested animals (q=3.2; P=0.032). These results

seems to indicate that c-fos is a good candidate for explaining the behaveoural effect of CaMK2N1 knockdown observed. This hypothesis will be discussed in detail at the Discussion section of this thesis.

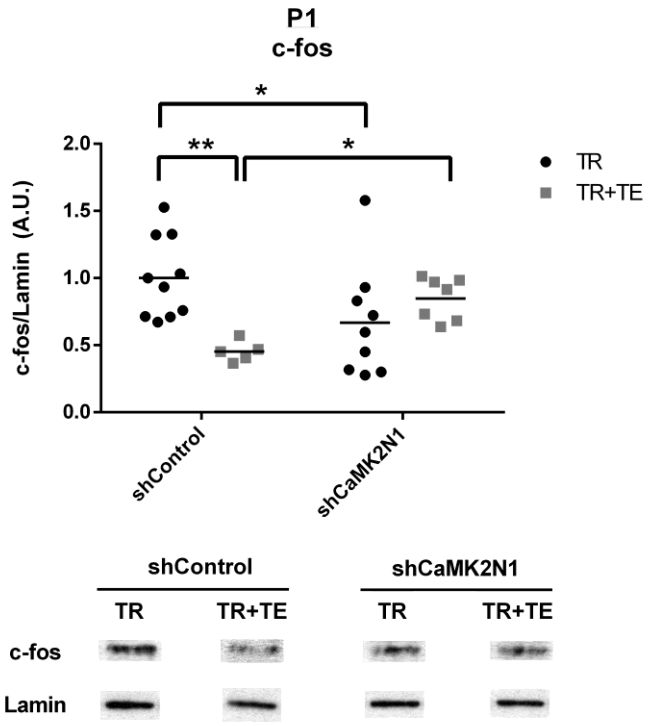

Figure 3.6-19 P1 c-fos levels after CaMK2N1 knockdown
The figure above shows the levels of c-fos in the P1 fraction of animals injected with shControl or shCaMK2N1 virus solution. Animals were also divided between those subjected to only training at CFC paradigm (TR) and those trained and tested for memory of context (TR+TE). Levels of c-fos were normalized by nuclear lamin. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show example bands of c-fos and Lamin for all groups. *P<0.05; **P<0.01

To further understand the effect of CaMK2N1 knockdown on c-fos expression we decided to look for any alterations of c-fos after CaMK2N1 knockdown on non-trained animals. This experiment was executed to look for baseline alterations in c-fos expression after CaMK2N1 knockdown. For this purpose samples from the experimental design explained on Figure 3.6-8 were used. One-way ANOVA showed no significant effect of CaMK2N1 knockdown on c-fos expression of naïve animals ($F_{1,13}$<0.001;

P=1.000) (Figure 3.6-20). This result indicated that the alterations in c-fos expression observed (Figure 3.6-19) are behaviourally induced.

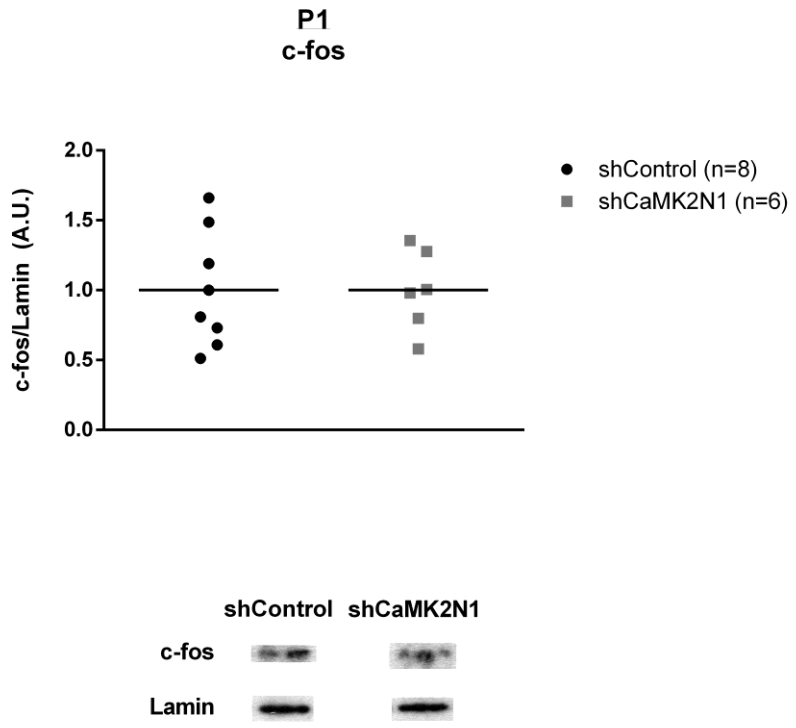

Figure 3.6-20 P1 c-fos levels after CaMK2N1 knockdown in naive animals
The figure above shows the levels of c-fos in the P1 fraction of naive animals injected with shControl or shCaMK2N1 virus solution. Levels of c-fos were normalized by the internal loading control, Lamin. The results were standardized to shControl average. Images on the bottom of the figure show example bands of c-fos and Lamin for all groups.

Guedea et al. (2011) have suggested that the phosphorylation of the transcription factor c-jun might regulate c-fos expression during memory extinction. Therefore, we analysed the levels of phosphorylated c-jun in the P1 fraction of our samples (Figure 3.6-21). Two-way ANOVA revealed no significant effect of virus treatment ($F_{1,30}$=0.2; P=0.637), of memory retrieval ($F_{1,30}$=2.6; P=0.116) and interaction effect ($F_{1,30}$=0.3; P=0.558) on phosphorylated c-jun levels.

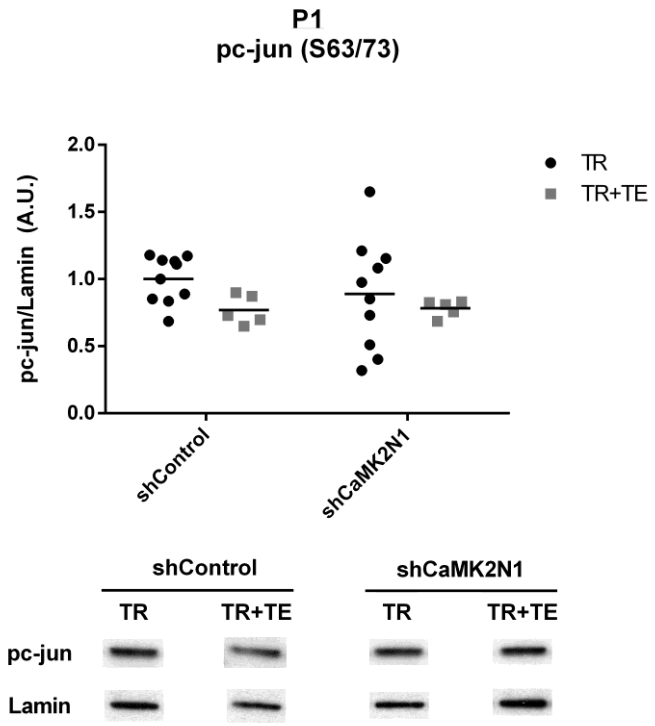

Figure 3.6-21 P1 phosphorylated c-jun levels after CaMK2N1 knockdown

This image shows the levels of phosphorylated c-jun in the P1 fraction of animals injected with shControl or shCaMK2N1 virus solution. Animals were also divided between those subjected to only training at CFC paradigm (TR) and those trained and tested for memory of context (TR+TE). Levels of pc-jun were normalized by nuclear lamin. The results of all the groups were standardized to TR shControl average. Images on the bottom of the figure show example bands of pc-jun and Lamin for all groups.

3.6.5 Effects of CaMK2N1 knockdown

Significant results obtain in the western blots and the behavioural studies performed are summarized in Table 3.6-1. These results are discussed in detail on the Discussion session of this thesis. Example membranes of the western blot analyses can be seen on Appendix "7.5 Example images of western blot membranes".

	shControl TR X TR+TE	**shCaMK2N1 TR X TR+TE**	**TR shControl X shCaMK2N1**	**TR+TE shControl X shCaMK2N1**
pCaMKII (T286)	↓			↑
GluA1				↑
pGluA1 (S831)				↓
c-fos	↓	↑	↓	
	shControl 24h X 4 days	**shCaMK2N1 24h X 4 days**	**24h shControl X shCaMK2N1**	**4 days shControl X shCaMK2N1**
Freezing scores		↓		↓

Table 3.6-1 Significant results from western blot and behavioural analyses
The table above summarize all the significant results obtain in the western blot and behavioural analyses. The significance of the results was obtained from SNK planned comparisons. First column shows the name of the dependent variable analysed. Second and third column show the significant effects of memory retrieval in shControl group and shCaMK2N1, respectively. The fourth and fifth column show the effects of CaMK2N1 knockdown with or without memory retrieval, respectively. Arrows indicate a significant result and its direction shows if the effect was an increase or decrease in the protein levels.

Chapter 4 Results with CaMK2N2 virus

4.1 Fluorescent images after viral vector transfection

Images presented on Figure 4.1-1 were taken from the brain of animals sacrificed 2 weeks after injection of viral solution containing the genes for CaMK2N2 an the fluorescent protein GFP. As can be observed the fluorescence is restricted to the hippocampus, indicating that the virus effectively transfected neuronal cells only in the hippocampus.

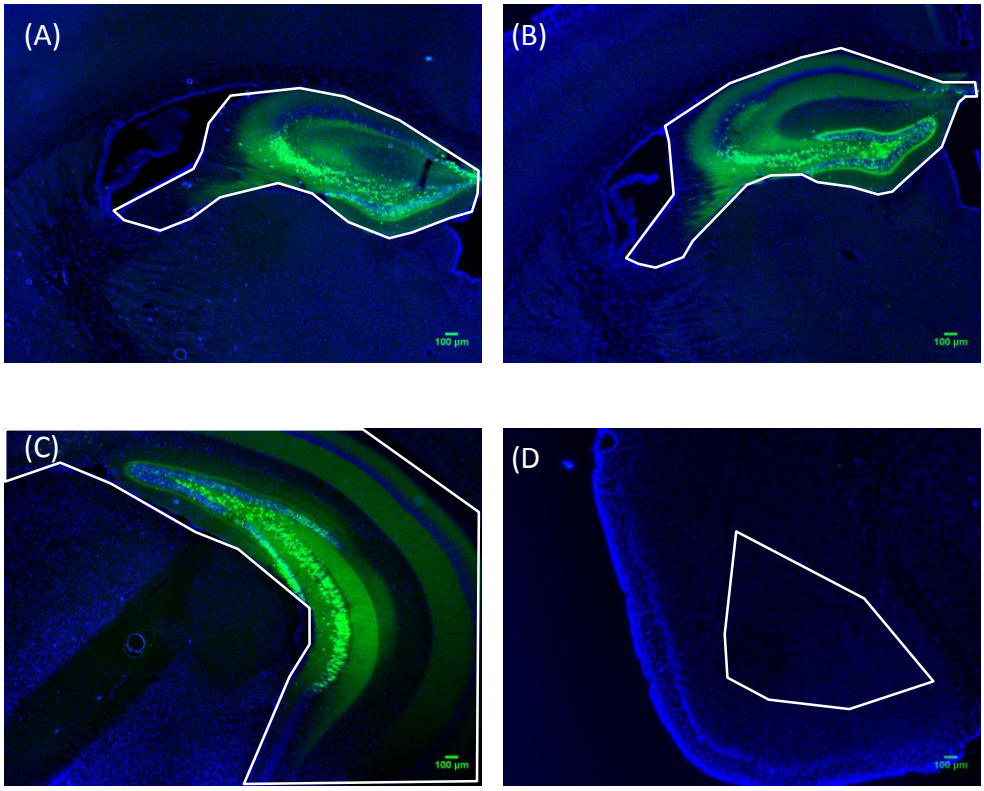

Figure 4.1-1 Fluorescent images for CaMK2N2 virus
Pictures of a mouse brain injected with CaMK2N2 viral solution. All the pictures show DAPI nuclear staining in blue and GFP fluorescence in green. Panels A to C show a sequence of images from the same hippocampus in an anterior-posterior order. The hippocampus is outlined inside the white line. Panel D shows an image of the amygdala of the same animal where one can notice the lack of GFP fluorescence. The amygdala is outlined inside the white line. Pictures were taken with a 2.5X objective and the scale can be seen on the bottom right corner.

4.2 Effect of CaMK2N2 overexpression on learning

The experimental design used to study the effect of hippocampal CaMK2N2 overexpression in the CFC paradigm was the same used for CaMK2N1 knockdown, presented on Figure 3.3-1. Briefly, animals were injected in both dorsal hippocampi, either with CaMK2N2 virus solution (CaMK2N2 virus) or control solution (Control virus). Two weeks later animals were placed in the conditioning chamber and received a mild foot shock, the US. Fear memory of the context was tested by re-exposure to the conditioning chamber without US for 5 minutes, 24 hours and 4 days after the conditioning session. Freezing score for animals from CaMK2N2 virus and Control virus groups are presented on Figure 4.2-1. Two-way ANOVA with repeated measures showed a significant effect of CaMK2N2 overexpression ($F_{1,19}$=4.7; P=0.043), significant effect of the memory tests ($F_{1,19}$=10.3; P=0.004), but no interaction ($F_{1,19}$=0.01; P=0.923). SNK planned comparisons showed a significant decrease of freezing behaviour in CaMK2N2 virus group when compared with Control virus group only in the 24 hours memory test (q=2.9; P=0.049), but not in the 4 days memory test (q=2.8; P=0.056). This result suggests that, different from CaMK2N1 knockdown, CaMK2N2 overexpression in the hippocampus causes a learning impairment phenotype. SNK planned comparisons also presented a significant effect of the memory tests within Control virus (q=3.4; P=0.027) and CaMK2N2 virus (q=3.0; P=0.044) groups.

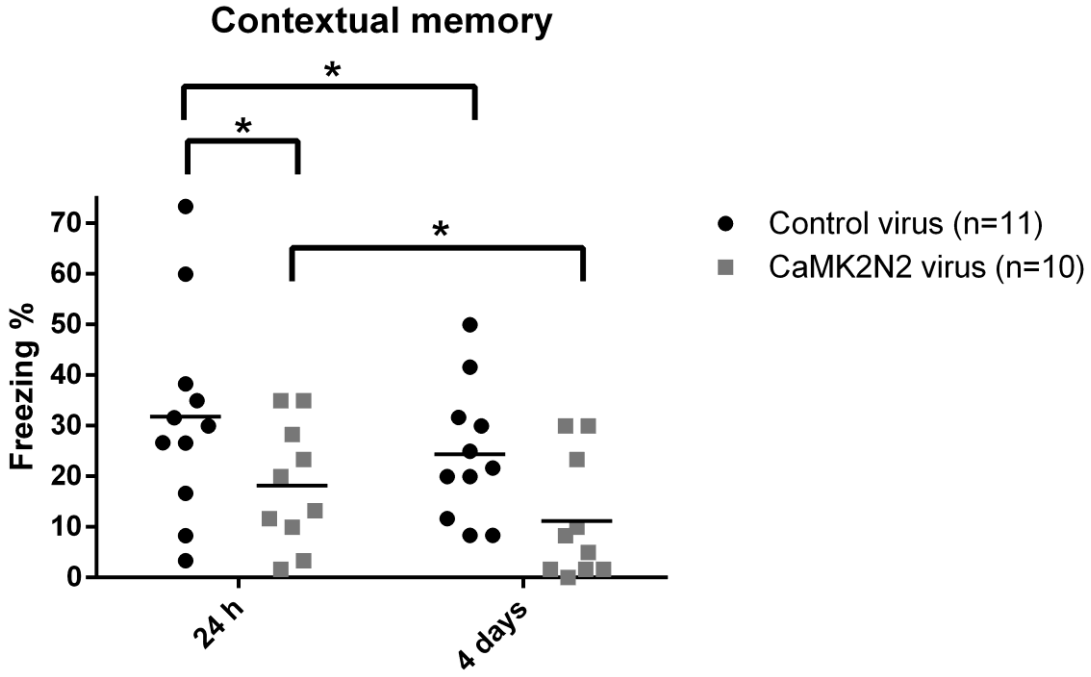

Figure 4.2-1 Freezing scores from experiment with CaMK2N2 overexpression
The graph above shows the average of freezing scores of animals from Control virus and CaMK2N2 virus group in the first (24h) and second (4 days) memory tests. Freezing scores are presented in the ordinate axis as percentage of freezing time in the 5 minutes of test. *P<0.05

To evaluate the efficiency and specificity of our overexpression treatment animals were sacrificed 2 hours after the last memory test, their dorsal hippocampus was dissected and used for RT-qPCR analyses of the mRNA levels of CaMK2N2 (Figure 4.2-2) and CaMK2N1 (Figure 4.2-3). One-way ANOVA test shows a significant increase in CaMK2N2 mRNA levels ($F_{1,20}$=48.6; P<0.001) with no effect on CaMK2N1 ($F_{1,20}$=3.1; P=0.093), confirming the efficiency and specificity of our virus treatment.

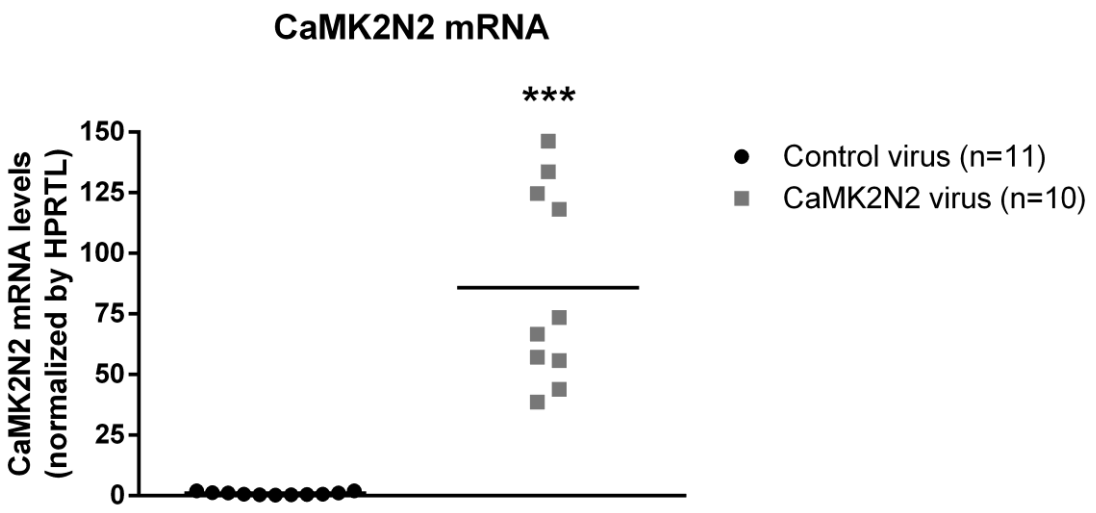

Figure 4.2-2 CaMK2N2 mRNA levels from experiment with CaMK2N2 overexpression
Quantitative analyses of the levels of CaMK2N2 mRNA. CaMK2N2 mRNA levels were normalized by HPRTL mRNA levels. All results were standardize to Control virus group average. *** P<0.001

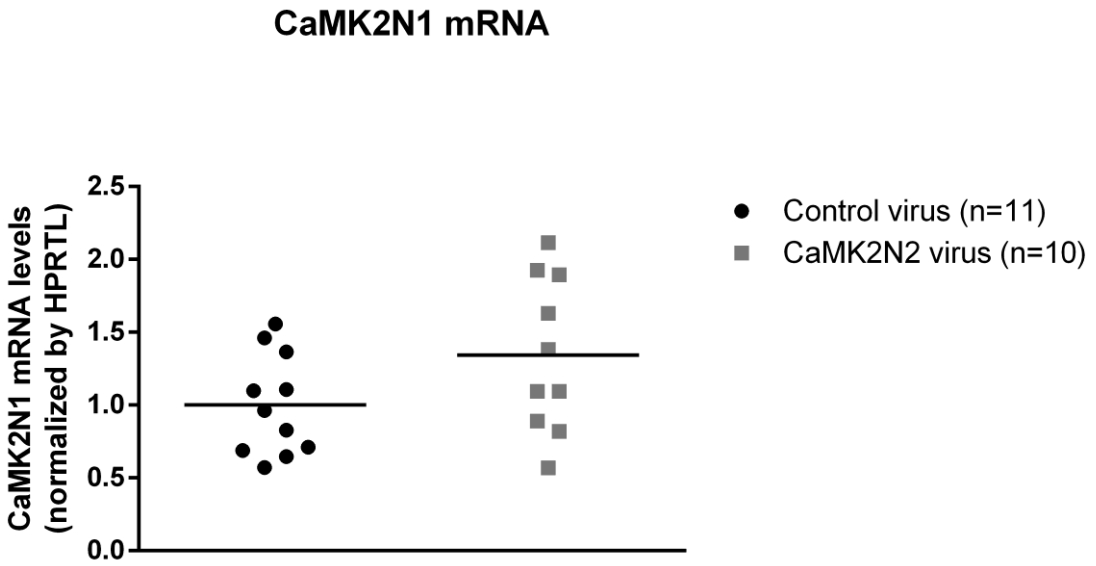

Figure 4.2-3 CaMK2N1 mRNA levels from experiment with CaMK2N2 overexpression
Quantitative analyses of the levels of CaMK2N1 mRNA. CaMK2N1 mRNA levels were normalized by HPRTL mRNA levels. All results were standardize to Control virus group average.

4.3 Effect of CaMK2N2 overexpression after training session

Considering that CaMK2N2 overexpression in the hippocampus caused a learning impairment (Figure 4.2-1) we decided to access the behavioural effect of CaMK2N2 overexpression after training in the CFC paradigm. To do so the experimental design presented on Figure 4.3-1 was used. In resume, animals were first trained in the CFC paradigm with one mild foot-shock. On the following day animals were injected intra-hippocampal with either Control or CaMK2N2 virus solution. Two weeks later animals were tested for the contextual memory followed by a second memory test 3 days later.

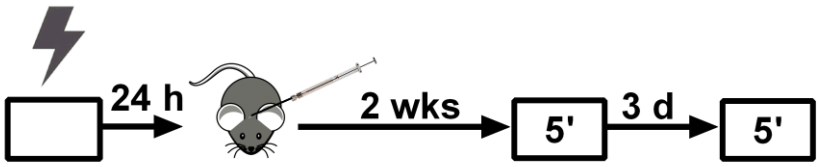

Figure 4.3-1 Experimental design CaMK2N2 overexpression after CFC training
The figure shows the experimental design for testing the effect of CaMK2N2 overexpression in the animals' freezing score after training in the CFC paradigm. Animals were first trained with one shock of 0.7 mA in the contextual chamber. One day later animals were injected with Control or CaMK2N2 virus solution. Two weeks later they were re-exposed to the context in two memory tests of 5 minutes each. The tests occurred 15 days and 18 days after training. Animals were sacrificed two hours after second memory test.

Freezing scores for animals from group Control virus and CaMK2N2 virus can be seen on Figure 4.3-2. Two-way ANOVA with repeated measures shows a significant effect of the repeated memory tests ($F_{1,13}$=39.1; P<0.001), but no effect of virus treatment ($F_{1,13}$=1.4; P=0.256) or interaction between these two factors ($F_{1,13}$=0.04; P=0.842). SNK planned comparisons shows a significant difference between the freezing scores on the first and second memory test in Control virus group (q=6.6; P<0.001) and CaMK2N2 virus group (q=5.8; P=0.001). No significant effect of CaMK2N2

overexpression was observed in the first (q=1.6; P=0.256) or second (q=1.4; P=0.322) memory tests.

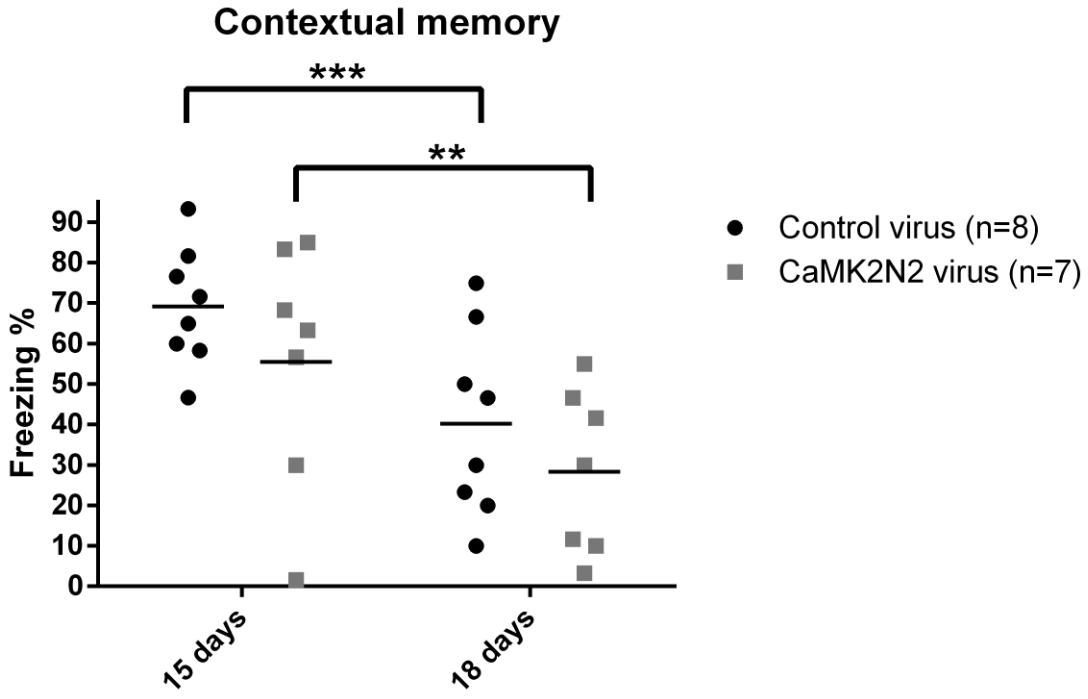

Figure 4.3-2 Freezing scores from experiment with CaMK2N2 overexpression after CFC training
The graph above shows the average of freezing scores of animals from Control virus and CaMK2N2 virus groups, in the first (15 days) and second (18 days) memory tests. Animals were treated in virus solution 24 hours after training in the CFC paradigm. Freezing scores are presented in the ordinate axis as percentage of freezing time in the 5 minutes of test. **P<0.01; ***P<0.001

RT-qPCR analyses of the dorsal hippocampus of the mice revealed a significant increase in the mRNA levels of CaMK2N2 ($F_{1,14}$=45.9; P<0.001) (Figure 4.3-3) with no significant effect on CaMK2N1 mRNA levels ($F_{1,14}$=0.5; P=0.468) (Figure 4.3-4).

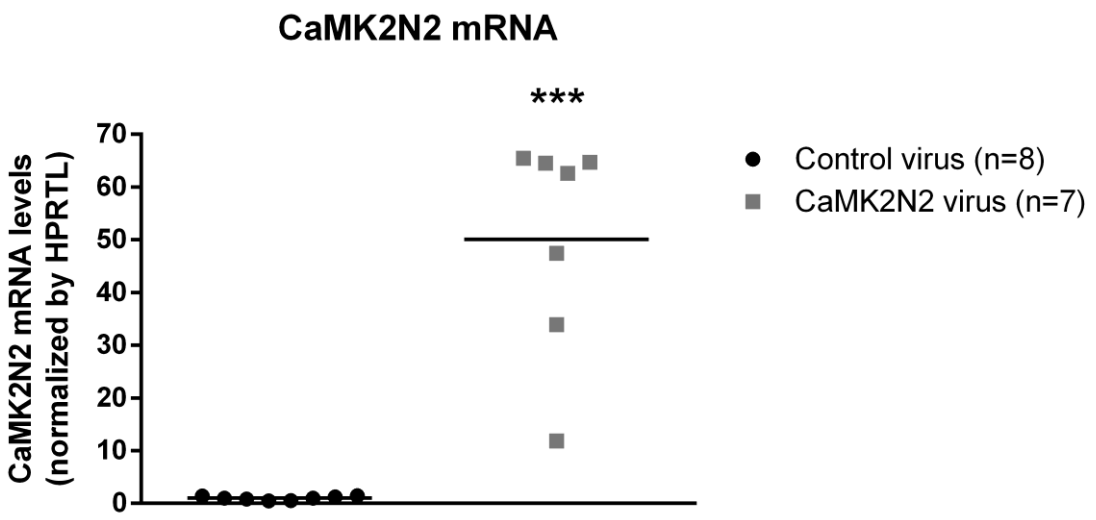

Figure 4.3-3 CaMK2N2 mRNA levels from experiment with CaMK2N2 overexpression after CFC training
Quantitative analyses of the levels of CaMK2N2 mRNA. CaMK2N2 mRNA levels were normalized by HPRTL mRNA levels. All results were standardize to Control virus group average. *** P<0.001

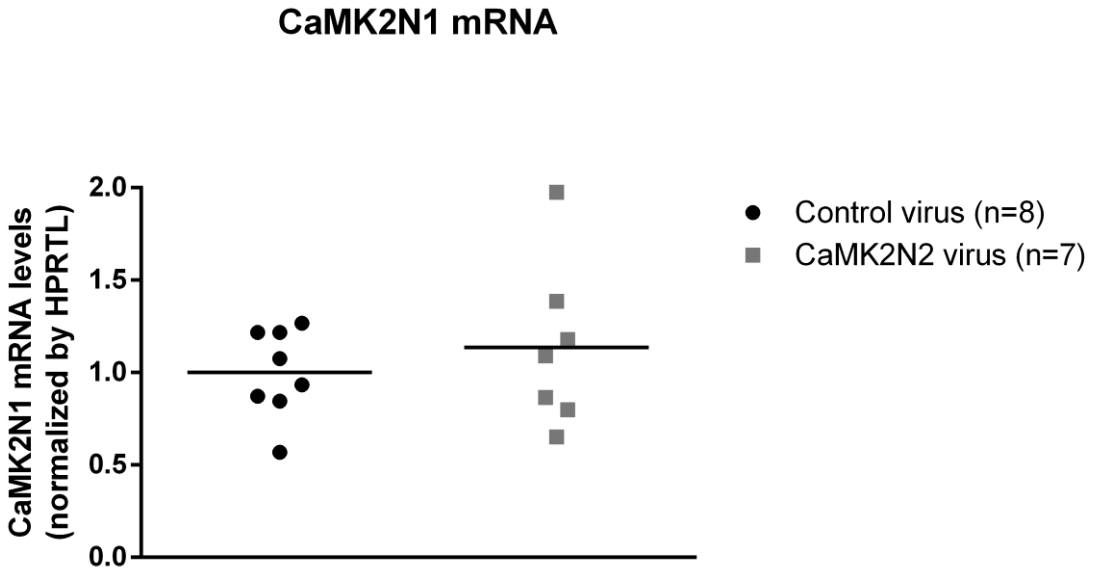

Figure 4.3-4 CaMK2N1 mRNA levels from experiment with CaMK2N2 overexpression after CFC training
Quantitative analyses of the levels of CaMK2N1 mRNA. CaMK2N1 mRNA levels were normalized by HPRTL mRNA levels. All results were standardize to Control virus group average.

4.4 Modelling CaMK2N2 overexpression

As presented on Figure 3.3-4, when we first knockdown CaMK2N1 we also observed a significant increase in CaMK2N2 mRNA levels of approximately 2X the control level. So far, the experiments presented here with CaMK2N2 overexpression caused an increase of 50-80X the level of the control. This increase is more than 10X the increase observed after CaMK2N1 knockdown, and unlikely to have the same behavioural effect. So, in order to study any possible behavioural effect of a smaller increase in CaMK2N2 we first did a dose-effect curve of our virus solution. The mRNA levels of CaMK2N2 in the dorsal hippocampus were measured in different animals after control virus injection or CaMK2N2 virus solution injection diluted 100X, 60X, 30X and 10X. The original CaMK2N2 virus solution was diluted with autoclaved PBS. As can be observed on Figure 4.4-1 the dilution of 60X was the one that presented an increase in CaMK2N2 mRNA levels closer to the increase observed after CaMK2N1 knockdown (see Figure 3.3-4 for comparison).

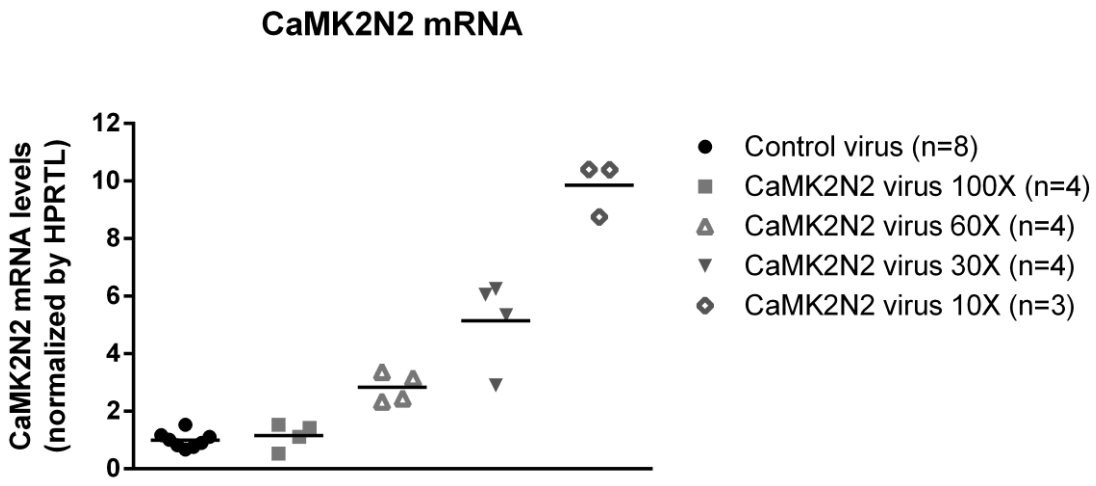

Figure 4.4-1 Dose-effect curve for CaMK2N2 virus solution
Quantitative analyses of CaMK2N2 mRNA levels after treatment with different dilutions of CaMK2N2 virus solution. CaMK2N2 mRNA levels were normalized by HPRTL mRNA levels. All results were standardize to Control group average.

To study the effects of the 60X diluted CaMK2N2 virus solution on mice behaviour at the CFC paradigm we used the experimental design represented on Figure 4.4-2. It was the same experimental design used to study the effect of CaMK2N1 knockdown. Briefly, mice were first injected with Control or CaMK2N2 60X virus solution. Two weeks later animals were trained in the CFC paradigm. One and four days later their fear memory was tested in the same context without the shock.

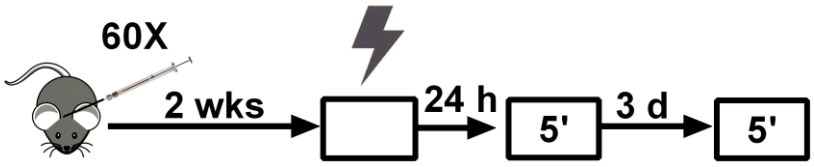

Figure 4.4-2 Experimental design 60X CaMK2N2 virus solution
The image shows the experimental design for testing the effect of a 60X diluted CaMK2N2 virus solution in the animals' freezing score in the CFC paradigm. Animals were first injected with Control or 60X CaMK2N2 virus solution. Two weeks later mice were trained with one shock of 0.7 mA in the contextual chamber. One day later they were re-exposed to the context in two memory tests of 5 minutes each. The tests occurred 24 hours and 4 days after the training session. Animals were sacrificed two hours after second memory test.

The freezing scores of animals from group Control virus and CaMK2N2 virus 60X can be seen on Figure 4.4-3. Two-way ANOVA with repeated measure showed a significant effect of memory test ($F_{1,22}$=11.2; P=0.003), but no effect of virus treatment ($F_{1,22}$=3.0; P=0.096) or interaction effect ($F_{1,22}$=0.3; P=0.589). SNK planned comparisons shows no significant effect for no comparison. There was no effect of the two memory tests within Control virus (q=2.9; P=0.051) or CaMK2N2 virus 60X (q=1.5; P=0.292) groups. There was also no effect of virus treatment in the first (q=0.5; P=0.713) or the second (q=0.9; P=0.523) memory test. This results indicates that the behavioural phenotype observed after CaMK2N1 knockdown (see Figure 3.3-2) was not caused by the consequent CaMK2N2 overexpression.

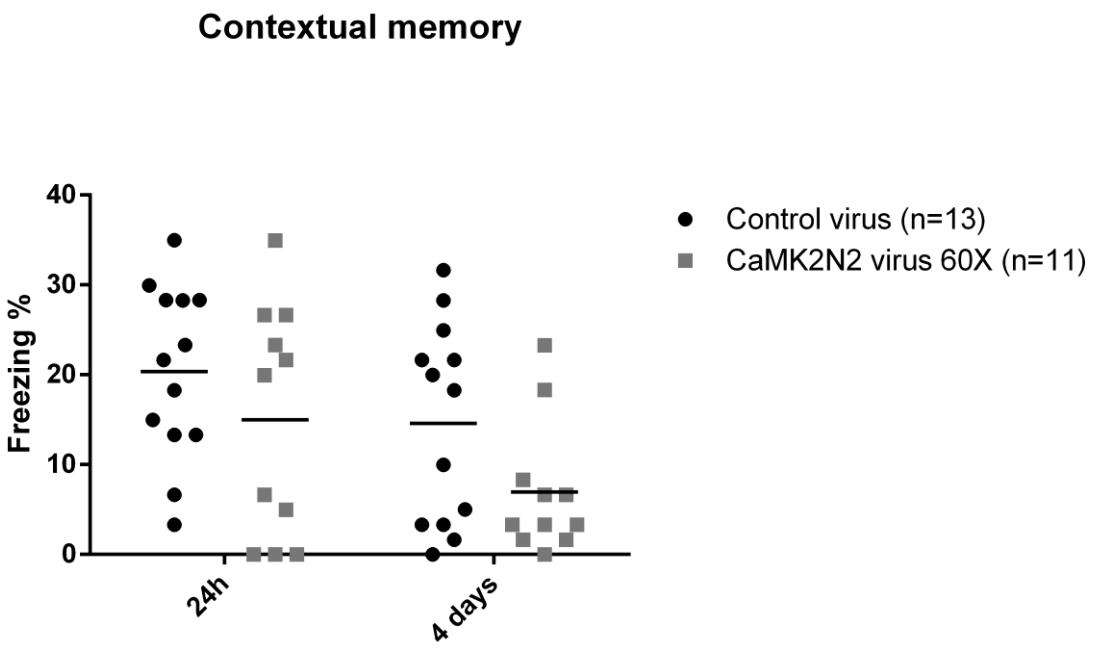

Figure 4.4-3 Freezing scores from experiment with CaMK2N2 virus solution diluted 60X
The graph above shows the freezing scores of animals from Control virus and CaMK2N2 virus 60X group in the first (24h) and second (4 days) memory tests. Freezing scores are presented in the ordinate axis as percentage of freezing time in the 5 minutes of test.

To confirm specificity and efficiency of the 60X diluted CaMK2N2 virus solution, 2 hours after the last memory test animals were sacrificed and their mRNA levels of CaMK2N2 (Figure 4.4-4) and CaMK2N1 (Figure 4.4-5), in the dorsal hippocampus, was measured by RT-qPCR. Treatment with 60X diluted CaMK2N2 virus solution caused a significant increase in CaMK2N2 mRNA levels of approximately 3X ($F_{1,20}$=81.1; P<0.001). No significant change was observed in the mRNA levels of CaMK2N1 ($F_{1,18}$=3.2; P=0.090).

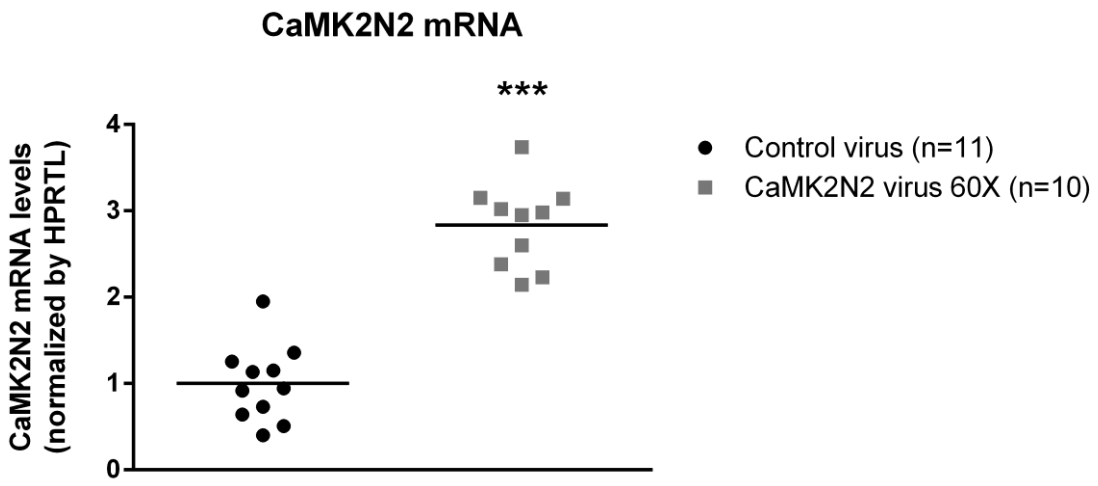

Figure 4.4-4 CaMK2N2 mRNA levels after treatment with 60X diluted CaMK2N2 virus solution
Quantitative analyses of the levels of CaMK2N2 mRNA. CaMK2N2 mRNA levels were normalized by HPRTL mRNA levels. All results were standardize to Control virus group average. *** P<0.001

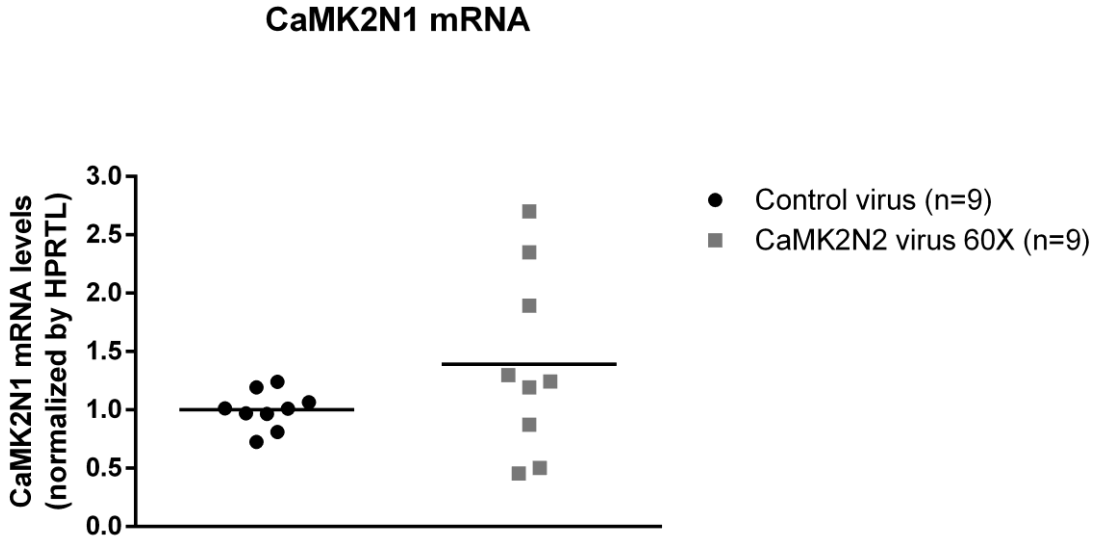

Figure 4.4-5 CaMK2N1 mRNA levels after treatment with 60X diluted CaMK2N2 virus solution
Quantitative analyses of the levels of CaMK2N1 mRNA. CaMK2N1 mRNA levels were normalized by HPRTL mRNA levels.

Chapter 5 Discussion

One of the aims of this thesis was investigate the function of the endogenous CaMKII inhibitors in CFC memory formation and maintenance. Our results show that changes in hippocampal levels of the inhibitors of CaMKII cause impairments in memory formation and maintenance. However the effects of knocking down or overexpressing an inhibitor of CaMKII are different. Knockdown of a CaMKII inhibitor reveals the relevance of the endogenous expression of this inhibitor in CFC learning/memory. This experimental approach tests whether the inhibitor needs to be normally expressed to fulfil a physiological function or if the inhibitor has a homeostatic function, a baseline effect. The overexpression of an inhibitor, distinctively, focuses on the functions of CaMKII in CFC learning and memory. Although, such approach also studies the effects of the inhibitor in CaMKII (effects in phosphorylation, kinase activity, and binding to other proteins) the behavioural effects of an overexpression of the inhibitor may not reflect the inhibitor's physiological function. Therefore, the results obtained for CaMK2N1 knockdown and CaMK2N2 overexpression will be discussed first separately in this thesis and then considered together. The last session of the Discussion will approach possible roles of CaMKII endogenous inhibitors in different neuropsychiatric disease.

5.1 CaMK2N1 knockdown

We have shown here for the first time that CaMK2N1 is required for maintenance of CFC memory after retrieval. Interestingly, this endogenous inhibitor of CaMKII is not required for memory formation or maintenance without retrieval. We have also shown that CaMK2N1 regulates the glutamatergic system and gene expression after retrieval. These observations will be discussed in detail bellow.

Our results indicate that CaMK2N1 expression in the dorsal hippocampus is increased after the retrieval of contextual fear memory. More specifically, after the retrieval of a weak contextual fear memory (3 shocks) (Figure 3.5-2), but not after retrieval of a stronger contextual fear memory (5 shocks) (Figure 3.5-5). Furthermore, we have shown that blocking of hippocampal CaMK2N1 expression results in memory impairment in a second memory test (Figure 3.3-2) but not in a first memory test executed either 24 hours (Figure 3.3-2) or 4 days (Figure 3.4-2) after CFC training. This implies that the physiological induction of CaMK2N1 expression, after memory retrieval, has a key role in CFC memory maintenance after retrieval.

Although based on our results we can conclude that the CFC memory impairment observed after hippocampal CaMK2N1 knockdown is dependent on a previous exposure of the animal to a first memory test, hence is retrieval-dependent, some specific details of this behavioural phenotype are yet to be defined. It is still unclear if the memory of a 5 shocks learning session will present the same behavioural phenotype after hippocampal CaMK2N1 knockdown. Our results on CaMK2N1 dorsal hippocampal expression seems to suggest that this might not be the case, since CaMK2N1 expression is not elevated after retrieval of a 5-shock-induced contextual fear memory. Nonetheless, more experiments are necessary to answer this question.

It is also unknown if the duration between the learning session and the first memory test has any relevance for the behavioural phenotype observed. Fukushima et al. (2014) observed that the amnesic effect of a systemic blocking of protein synthesis, after retrieval of an inhibitory avoidance memory, can be achieved with memory retrieval sessions performed 1 day or 3 days after learning session. In the case of CFC, Trent et al. (2015) reported an amnesic effect of zif268 blocking in the dorsal hippocampus due to memory retrieval session performed 2 days after training session. Furthermore, intra-amygdalar injection of anisomycin has an retrieval-induced amnesic effect, on tone fear conditioning memory, even for memories retrieved 14 days after training session (Nader et al., 2000). However, Hall et al. (2001) observed that retrieval of CFC memory induced an increase in hippocampal CA1 Zif268 expression only for retrieval of a 24 hours memory but not a 28 days old memory. Additionally, systemic treatment with anisomycin impaired inhibitory avoidance memory only if the first memory test was executed 1 day or 7 days after training but not if it was executed 14 days or 24 days after training (Milekic and Alberini, 2002). It has also been reported that different time points for tone fear memory retrieval session will induce an increase in c-fos expression in different circuits within the same subregion of the brain (Do-Monte et al., 2015). For example, the basolateral nucleus of the amygdala showed a significant increase in c-fos expression if retrieval session occurred 6 hours or 24 hours, but not 7 days, after training. The central nucleus of amygdala, on the other hand, showed an increase of c-fos expression only if memory was retrieved 7 days after tone fear memory training session (Do-Monte et al., 2015). Is still unclear if such activation changes within subregions of the hippocampus can also be observed due to different time points for the retrieval of a contextual fear memory. More experiments are also necessary to

determine the relevance of temporal variations on the memory retrieval session for the behavioural phenotype observed in this thesis.

Another important future experiment would be to test this retrieval-induced memory impairment in a different behavioural paradigm. As discussed by Fukushima et al. (2014), the inhibitory avoidance test is a good test to study retrieval induced memory effects. When testing memory retrieval in most paradigms, as for example in the CFC, re-exposure of the animal to the context can induce memory reconsolidation but it can also induce memory extinction since the animal is exposed to the CS (the context) without the US (shock). However during training in the inhibitory avoidance task the animal is first placed in a light compartment and only when it enters the dark compartment it receives a shock. Therefore, if we test the animal after conditioning by just placing it in the light compartment the context would induce reconsolidation without memory extinction, since the animal would not have any contact with the dark compartment (Fukushima et al., 2014). It would be interesting to test the behavioural phenotype observed after hippocampal CaMK2N1 knockdown in the inhibitory avoidance paradigm proposed by Fukushima et al. (2014).

To better understand the downstream molecular effects of hippocampal CaMK2N1 knockdown we analysed the total levels and phosphorylation levels of different proteins by western blot technique. Our first set of western blots looked at the effects of CaMK2N1 knockdown in total αCaMKII levels and T286 phosphorylation. Our results seems to indicate that CaMK2N1 plays a major role in regulating αCaMKII T286 phosphorylation within the synapses (Figure 3.6-3) with no significant effect of CaMK2N1 knockdown in T286 phosphorylation in cytosolic fraction (Figure 3.6-5). Although not significant, a trend of an effect of virus treatment ($F_{1,24}$=4.037; P=0.058) in

cytosolic αCaMKII T286 phosphorylation levels was observed (Figure 3.6-5). Been that the case, it is in the opinion of this author that more studies are needed to clarify if CaMK2N1 plays any role in regulation of cytosolic αCaMKII phosphorylation. The analyses of samples collected in different time points after memory retrieval and the utilization of different analysis techniques (for example immunohistochemistry) would be important next steps. Regarding αCaMKII T286 phosphorylation at the synapses, a significant decrease in T286 phosphorylation levels after memory retrieval in shControl group animals, but not in shCaMK2N1 animals was found (Figure 3.6-3). In fact, after one memory test αCaMKII T286 phosphorylation levels in shControl animals were significantly lower to αCaMKII T286 phosphorylation in shCaMK2N1 animals (Figure 3.6-3). This result shows for the first time that a decrease in T286 phosphorylation is triggered by CFC memory retrieval. We also observed in a separate experiment (Figure 3.5-1) an increase in the expression of CaMK2N1 mRNA after CFC memory retrieval (Figure 3.5-2). It is likely that the decrease in T286 phosphorylation observed after memory retrieval in one experiment is related to the increase in CaMK2N1 mRNA levels observed after retrieval in the other experiment. This hypothesis is corroborated by the lack of changes in αCaMKII T286 phosphorylation levels of shCaMK2N1 animals after memory retrieval (Figure 3.6-3).

Our second set of western blot analyses was focus at a known CaMKII substrate, the sub-unit GluA1 of the AMPAR. We observed that the total levels of GluA1 in the P2 fraction were significantly higher in shCaMK2N1 group compared with shControl group, only if fear memory was retrieved (Figure 3.6-6). Animals from shControl group had a retrieval-induced decrease in synaptosomal GluA1 levels when compared to shCaMK2N1 group (Figure 3.6-6). With a similar experimental design from ours, Rao-

Ruiz et al. (2011) also observed a decrease of synaptosomal GluA1 levels in the dorsal hippocampus, 1 hour after retrieval of CFC memory (executed 24 hours after training). Additionally, they also reported this decrease to be transient, with synaptosomal levels of GluA1 been normal 4 or 7 hours after memory retrieval session (Rao-Ruiz et al., 2011). Rao-Ruiz et al. (2011) hypothesis is that a decrease AMPAR at the synapses would be induced transiently after memory retrieval, given a momentary liability to the memory. It is known that CaMKII activation, trough pharmacological activation of NMDAR, stabilizes AMPAR localization in the PSD (Opazo et al., 2010). ShControl animals presented a retrieval-induced reduction on synaptosomal GluA1 levels (Figure 3.6-6) but also a retrieval-induced decrease in CaMKII T286 phosphorylation levels (Figure 3.6-3). These results, taken together with Opazo et al. (2010) observation, suggests that CaMKII inactivation after memory retrieval is necessary to block any activity induced increase in AMPAR synaptosomal levels. Corroborating with this hypothesis, CaMK2N1 knockdown resulted in higher levels of synaptosomal CaMKII T286 phosphorylation (Figure 3.6-3) and GluA1 levels (Figure 3.6-6) after retrieval of a memory. This results is in agreement with Opazo et al. (2010) observation that increasing CaMKII inhibition by CN21 treatment blocks NMDAR-induced stabilization of AMPR in the PSD (Opazo et al., 2010). Furthermore, it also suggests that the retrieval-induced memory labialization due to a reduction on AMPAR synaptosomal levels observed by Rao-Ruiz et al. (2011) might be regulated by CaMK2N1. In terms of GluA1 phosphorylation at S831, a significant decrease in GluA1 phosphorylation after CaMK2N1 knockdown was observed only in animals subjected to the memory test (Figure 3.6-7). Recent observations made by Hosokawa et al. (2015) have shown that only a minor percentage of all the GluA1 subunits in the synapses seems to be phosphorylated at serine 831. Therefore, is unlikely that this phosphorylation plays any crucial role in memory maintenance. We also

quantified total GluA1 and S831 phosphorylation in naïve animals treated with shControl or shCaMK2N1 virus solutions. No significant effect of CaMK2N1 knockdown was observed in total GluA1 levels (Figure 3.6-9) and levels of phosphorylation at S831 (Figure 3.6-10) if animals were not subjected to any behavioural paradigm, suggesting that CaMK2N1 does not play a role in GluA1 regulation during basal activity. More experiments are necessary to also study the phosphorylation of GluA1 at serine 567. CaMKII has been show to phosphorylate GluA1 S567 after the induction of LTD, resulting in a decrease in the number of AMPAR within the synapses (Coultrap et al., 2014; Lu et al., 2010).

It is also known that inhibition of CaMKII with CaMKIINtide decreases total GluA1 levels by regulation of protein degradation (Naskar et al., 2014). Furthermore, CaMKII has been shown to regulate the activation of the proteasome system (Djakovic et al., 2009; Jarome et al., 2013), its localization in the PSD (Bingol et al., 2010) and polyubiquitination of proteasomal targets (Thein et al., 2014). Hence, our next set of western bots analysed protein degradation and the proteasome system. The scaffolding protein Shank has been shown to be degraded 2 hours after CFC memory retrieval session (Lee et al., 2008). However, no significant effect of CaMK2N1 knockdown in the levels of Shank protein (Figure 3.6-11) was observed in our experiments. Polyubiquitination of proteins has also been observed to be increased after memory retrieval (Fukushima et al., 2014; Lee et al., 2008). Again, no significant effect of CaMK2N1 knockdown was observed in the levels of ubiquitinated proteins (Figure 3.6-12). Finally we also quantified total levels (Figure 3.6-13) and phosphorylation at S120 of the proteasomal subunit Rpt6 (Figure 3.6-14). CaMKII has been showed to phosphorylate Rpt6 causing an increase in proteasomal activity (Djakovic et al., 2009;

Jarome et al., 2013). Nonetheless, no significant effect of CaMK2N1 knockdown was observed in Rpt6 phosphorylation (Figure 3.6-14) or total levels (Figure 3.6-13) in our experiment. Taken together, these results suggest that the behavioural phenotype observed due to CaMK2N1 knockdown was not related to protein degradation. Despite this, more studies are necessary to clarify if CaMK2N1 plays any role in the regulation of protein degradation. It has not been addressed in this thesis, for example, the molecular effects of our treatment immediately after the training session, or at different time points after memory retrieval. The lack of any commercially available antibody against phosphorylated Rpt6 also presents an obstacle for more studies.

Our final set of western blot analyses concentrated on the analysis of molecules that regulate gene expression. Gene expression has been shown to be a necessary process for memory maintenance after retrieval (Kelly et al., 2003; Lee et al., 2004). No significant changes on total levels of the transcription factor CREB (Figure 3.6-16) and its phosphorylation at S133 (Figure 3.6-17) were observed 1 hour after memory retrieval with or without CaMK2N1 knockdown.

We also quantified the nuclear levels of Zif268 (Figure 3.6-18), a known biological substrate of memory reconsolidation (Lee, 2008; Lee et al., 2004). Although two-way ANOVA test showed a significant effect of interaction between virus treatment and memory test ($F_{1,33}$=5.776; P=0.023), no significant result was obtained with SNK planned comparisons. Since the interaction effect observed was not maintained after planned comparison not much conclusion can be draught from this data. The higher tendency of effect observed after planned comparisons was in the comparison between trained (TR) and trained and tested (TR+TE) animals from shCaMK2N1 group (q=2.575; P=0.079). Further experiments are necessary to understand any possible retrieval-dependent

effect of hippocampal CaMK2N1 knockdown on Zif268 expression. An important next experiment would be the analyses of Zif268 expression within different subregions of the hippocampus. Using *in situ* hybridization technique, Hall et al. (2001) reported an increase in Zif268 expression only in hippocampal CA1 area but not in the DG, after retrieval of CFC memory. Lee et al. (2004) observed, by western blot analyses, a CFC memory retrieval-induced increase in Zif268 expression of 200% in CA1 area, but only a 50% increase in DG/CA3 areas. Furthermore, Lee et al. (2004) also showed that blocking of Zif268 expression in hippocampal CA1 area, before retrieval session, is sufficient to impair CFC memory reconsolidation.

Another regulator of gene expression analysed was c-fos. C-fos is known to be a biological substrate of memory consolidation (Huff et al., 2006) and extinction (Do-Monte et al., 2010; Guedea et al., 2011; Plendl and Wotjak, 2010; Tronson et al., 2009). However, very little is known about what is the function of c-fos expression in retrieval-dependent memory maintenance or extinction. Our results show a significant decrease in c-fos expression in trained only animals (TR) when comparing shCaMK2N1 animals to shControl animals (Figure 3.6-19). In this case, CaMK2N1 seems to be important for maintaining c-fos expression after CFC training session. Nonetheless, the lack of a memory impairment in shCaMK2N1 animals during the first memory test (Figure 3.3-2) indicates that the animals had normal learning and consolidation of the CFC task. Hippocampal c-fos expression has been shown to be up-regulated 1 hour after CFC training session (Huff et al., 2006), but it is unknown if any CFC training-induced alteration in hippocampal c-fos expression would still be significant at the time point used in our experiments, 25h after training session. Any training-induced c-fos expression alteration caused by CaMK2N1 knockdown in our experiment seems to have

not resulted in a behavioural phenotype. von Hertzen and Giese (2005) proposed the existence of a greater functional redundancies during consolidation than during reconsolidation. This greater function redundancy of memory consolidation may explain why Lee et al. (2004) saw a reconsolidation impairment but not a consolidation impairment after blocking of hippocampal Zif268 expression, even though Zif268 has been shown to be involved in consolidation and reconsolidation of CFC task (Hall et al., 2001; Hall et al., 2000). The possibility of less redundancy in CFC memory reconsolidation may also explain why, although CaMK2N1 knockdown impacts on c-fos expression both after training and after testing there was an impairment in memory only after memory retrieval. No significant effect of CaMK2N1 knockdown in dorsal-hippocampal on c-fos expression was observed in naïve animals. Therefore, it is likely that hippocampal CaMK2N1 does not regulate basal expression of c-fos.

A significant reduction in dorso-hippocampal c-fos expression of shControl animals after memory retrieval was observed. ShCaMK2N1 animals, on the other hand, had an increase in c-fos expression after memory retrieval (Figure 3.6-19). This seems to indicate that CaMK2N1 is important for inhibition of c-fos expression 1 hour after a first memory test, and this inhibition might be important for memory maintenance. The opposite direction of the effects of CaMK2N1 knockdown if the animals are just trained or if they are trained and tested (Figure 3.6-19) implies that CaMK2N1 might play different roles during memory formation and memory maintenance. The retrieval-dependent increase in c-fos expression, observed after CaMK2N1 knockdown, could be related to the retrieval-dependent memory impairment observed previously (Figure 3.3-2). That being the case, one would assume that a decrease in hippocampal c-fos expression, 1 hour after memory retrieval, would be important for memory

maintenance. At first this hypothesis might seem a little different from the usual accepted functions of c-fos. For example, hippocampal c-fos expression has been shown to be down-regulated after fear memory extinction (Guedea et al., 2011; Tronson et al., 2009). Nonetheless, shControl animals presented a decrease in hippocampal c-fos expression 1 hour after memory retrieval (Figure 3.6-19). To make sense of this apparent contradiction it is important to analyse the role of c-fos expression within the different subregions of the hippocampus. For example, Fukushima et al. (2014) observed an increase in c-fos expression, after inhibitory avoidance memory retrieval, in hippocampal CA1 and CA3 areas but no in the DG. Furthermore, different regulation of c-fos expression in the CA1 and DG can also be observed during tone fear memory extinction. While CA1 c-fos expression is increased after tone memory extinction in the trained context or in a novel context, DG c-fos levels are increased only if the tone memory is extinguished in the trained context (Knapska and Maren, 2009). Regarding the CFC paradigm, Wheeler et al. (2013) reported a retrieval-induced increase in CA1 c-fos expression, but a decrease in c-fos expression within the DG. With a similar experimental design from the one used in this thesis, Besnard et al. (2014) saw that 1 hour after 5 min retrieval session, executed 24 hours after a previous session of contextual fear conditioning with one 0.7 mA shock, there was a significant decrease in DG c-fos expression when comparing animals only trained and trained and tested animals. The significant decrease in c-fos expression observed in this thesis within shControl animals after CFC memory retrieval (Figure 3.6-19) is in accordance with the retrieval-induced decrease in c-fos expression observed within the DG on the experiments performed by Besnard et al. (2014) and Wheeler et al. (2013). The samples probed in our western blot analyses are of dorsal hippocampus, which will include different hippocampal subregions. However, it is known that the DG contains

aproximatly 5X more neurons then CA1 or CA2/CA3 areas, accounting for the majority of neurons in the hippocampus (West et al., 1991). Therefore, it is likely that the western blot analyses presented in this thesis are more representative of the hippocampal surbregion DG than of the other hippocampal subregions. This hypothesis is corroborated by the similarity of our observations with the retrieval-induced alterations in c-fos expression observed within the DG neurons by Besnard et al. (2014) and Wheeler et al. (2013). To further test this hypothesis an important next step would be to quantify c-fos expression in the different subregions of the hippocampus separately. The necessity of a separate analysis for the DG and CA1 areas is reinforced by the different dependence of αCaMKII T286 autophosphorylation for LTP induction between this areas. Whilst CA1 glutamatergic neurons required αCaMKII T286 autophosphorylation for induction of NMDAR-dependent LTP (Giese et al., 1998), DG glutamatergic neurons does not (Cooke et al., 2006).

Taken together, the western blot analyses of αCaMKII T286 phosphorylation (Figure 3.6-3), synaptosomal GluA1 levels (Figure 3.6-6) and nuclear c-fos expression (Figure 3.6-19) we observed a similar effect of decrease of these three variables after memory retrieval in control animals. In contrast, animals from shCaMK2N1 group show a retrieval-induced increase in the levels of αCaMKII T286 phosphorylation (Figure 3.6-3), synaptosomal GluA1 levels (Figure 3.6-6) and nuclear c-fos expression (Figure 3.6-19). Based on this observation we raise the hypothesis that retrieval-induced expression of CaMK2N1 causes a decrease in neuronal activity that seems to be important for memory maintenance. A schematic representation of this hypothesis can be seen on Figure 5.1-1.

It is our hypothesis that this inhibition of CaMKII after memory retrieval happens on the DG, where the inhibition of CaMKII activity would be important to block a new memory from be acquired. Been that the case, the endogenous inhibitors of CaMKII would play a key role in this retrieval-induced blocking of CaMKII. This hypothesis is been proposed for the first time in this thesis, so it is necessary to further investigate this question to consolidate this hypothesis. In the CA1 area of the hippocampus this might not be the case. It has been well established that after memory retrieval this area is activated, with an increase in transcription factors that is necessary to keep the memory (Hall et al., 2001; Lee et al., 2004; Wheeler et al., 2013). The function of the CaMKII endogenous inhibitors after memory retrieval in the CA1 area is still unknown. Furthermore, one should consider that in different time points the activation or inhibition of CaMKII is likely to change.

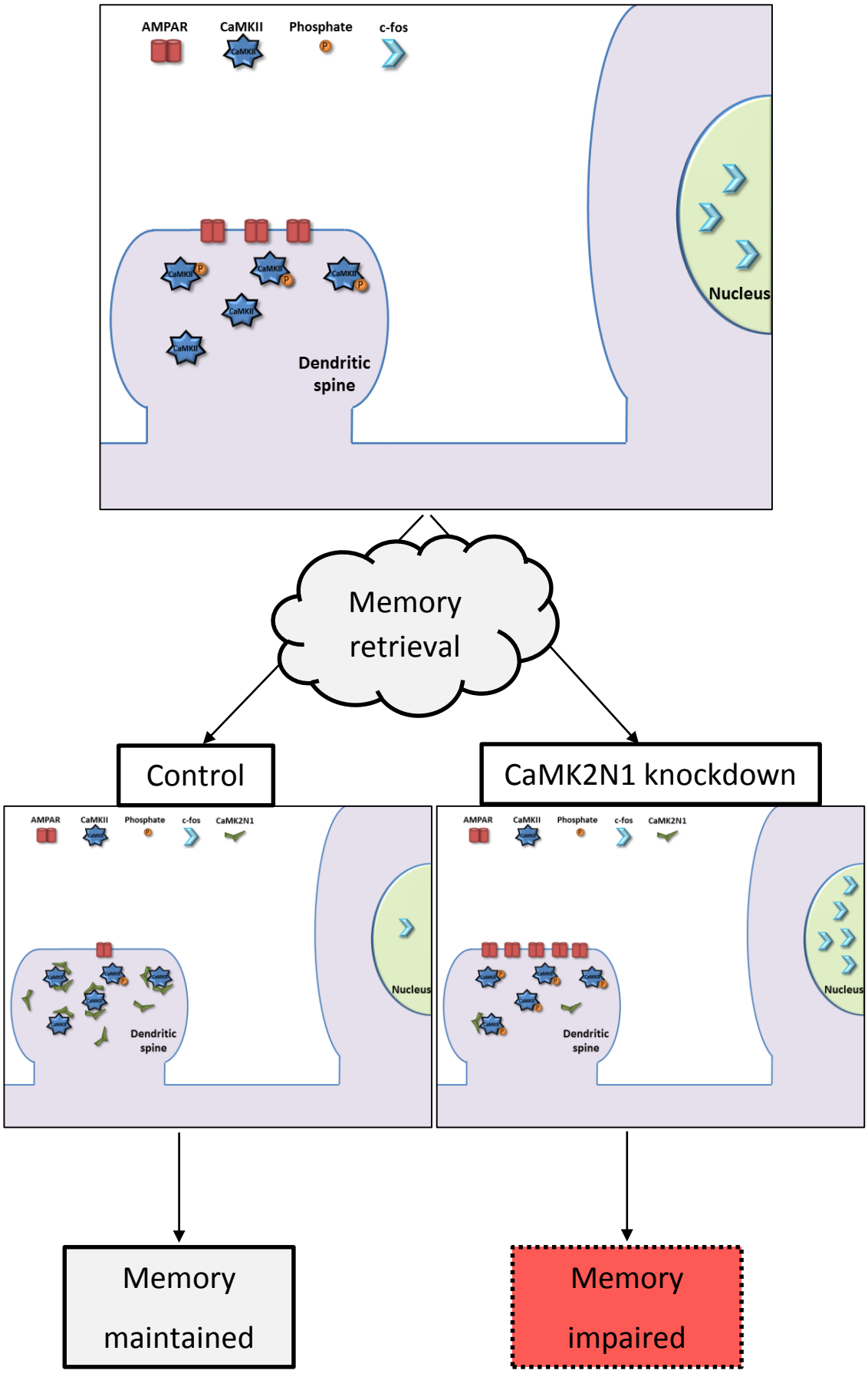
AMPAR
CaMKII
Phosphate
c-fos
Nucleus
Dendritic spine
Memory retrieval
Control
CaMK2N1 knockdown
CaMK2N1
Memory maintained
Memory impaired

Figure 5.1-1 CaMK2N1 function after CFC memory retrieval
The schematic figure above summarizes the retrieval-induced molecular and behavioural effects observed after hippocampal CaMK2N1 knockdown. The only significant retrieval-induced effects of CaMK2N1 knockdown not presented in this scheme are in Glua1 S831 phosphorylation (Figure 3.6-7) and in Zif268 expression (Figure 3.6-18). Zif268 alterations were not represented in this figure due to the fact that no significant effect was seen after planned comparisons. GluA1 S831 phosphorylation has been shown to be presented in only a minor percentage of GluA1 subunits in the synapses (Hosokawa et al., 2015). Therefore, is unlikely that this effect is related to the behavioural phenotype observed and it was not represented in this scheme. The scheme presents the results according to the following hypothesis. After CFC memory retrieval hippocampal glutamatergic neurons (likely from the DG) have an increase in CaMK2N1 expression. CaMK2N1 will block activation of CaMKII within the synapse, with consequent decrease in synaptosomal AMPAR and nuclear c-fos levels. This process is necessary for memory maintenance and is likely to be transient. After hippocampal CaMK2N1 knockdown the lack of CaMKII inhibition resulted in an increase in CaMKII T286 autophosphorylation, synaptosomal GluA1 levels and c-fos nuclear levels. This excessive activation after memory retrieval resulted in memory impairment in animals from shCaMK2N1 group. Molecules presented in the images of this scheme are identified on the top left corner of every image.

This dependence of memory maintenance to a decrease in neuronal activity, likely to be in the DG, after CFC memory retrieval could be interpreted by two different perspectives. One possibility is that the retrieval-induced memory impairment observed after CaMK2N1 knockdown is an increase in memory extinction. In other words, CaMK2N1 knockdown resulted in a quicker learning of the new environmental conditions, the box context no longer comes associated with the shock, thus suppressing the previously learned CR in a second memory test. Therefore, the retrieval induced reduction in neuronal activity observed in the shControl group maintains the fear memory by inhibiting the extinction of the associative memory between the US and the CS. Such hypothesis is corroborated by Plendl and Wotjak (2010) observation that between-session extinction of tone fear memory was accompanied by an increase in the number of c-fos-positive neurons in the DG of mice. Furthermore, Santini et al. (2004) have shown that there is an increase in c-fos expression at the medial prefrontal cortex after extinction and blocking of protein synthesis in this region does not impair the effect of one memory extinction trial but blocks the accumulative effect of repetitive extinction trials. Based on this observations Santini et al. (2004) proposed that c-fos function in extinction is to consolidate the extinction memory. By this interpretation the amnesic

effect of CaMK2N1 knockdown would be understood as an increase in memory extinction due to the increase in c-fos expression.

The retrieval-induced memory impairment observed after CaMK2N1 knockdown could also be interpreted as a destabilization impairment. Part of the memory reconsolidation hypothesis, destabilization of a memory has been shown to be a necessary process for memory maintenance after retrieval (Artinian et al., 2008). We could interpret the decrease in αCaMKII T286 phosphorylation (Figure 3.6-3), synaptosomal GluA1 levels (Figure 3.6-6) and nuclear c-fos expression (Figure 3.6-19) observed in control animals as part of the destabilization of a memory. That been the case, we could expect that the increase in the same factors observed in shCaMK2N1 group would result in a retrieval-induced memory impairment due to blocking of memory destabilization. It is also too early to discard the possibility of reconsolidation impairment. The reconsolidation of a memory after destabilization requires the synthesis of new proteins (Lee et al., 2008). At the time point observed in our experiments, control animals showed no retrieval induced increase in the expression of any of the transcription factor quantified in this thesis, but rather a retrieval induced decrease. This implies that these molecules at this time point are not a good target for studying memory re-stabilization. However the analyses of other molecules and different time points might show different results. The first step to study any possible re-stabilization impairment in CaMK2N1 knockdown animals is to find a molecule that has a retrieval induced increase in control animals subjected to our experimental conditions.

Nevertheless, the clear separation of any retrieval-induced amnesic phenotype between a reconsolidation impairment and an increased memory extinction is still a

matter of debate. Reconsolidation impairment and memory extinction differ from each other by their molecular bases, like the specificity of Zif268 to reconsolidation (Lee et al., 2004), and their behavioural peculiarities, like the possibility of reinstatement only in extinguished memories (Rescorla and Heth, 1975). However, Trent et al. (2015) reported the reinstatement of CFC memory impaired by hippocampal Zif268 blocking. Raising the question if extinction and memory reconsolidation are two independent processes or are only two different outcomes of the same process. Based on the similarity of molecules shown to be relevant for memory reconsolidation and extinction, Almeida-Correa and Amaral (2014) also proposed a common biological substrate for both process. As mentioned by Almeida-Correa and Amaral (2014) molecules like the cannabinoid receptor type 1, the ubiquitin-proteasome system, the sub-unit GluA2 of the AMPAR and others, have been shown to be relevant for memory extinction (Dalton et al., 2008; Marsicano et al., 2002; Pick et al., 2013; Suzuki et al., 2004) and for retrieval-induced memory destabilization (Hong et al., 2013; Lee et al., 2008; Suzuki et al., 2008). It is also known that a memory reconsolidation session facilitates the extinction of the same memory. For example, a 3 minutes retrieval session (capable of inducing reconsolidation but not extinction) executed 1 hour before memory extinction session, has been shown to enhance extinction reducing spontaneous or induced recovery of cued fear memory (Monfils et al., 2009). Similar observation have also been made for contextual fear memory (Rao-Ruiz et al., 2011). Almeida-Correa and Amaral (2014) also based their hypothesis of a common mechanism for memory reconsolidation and extinction on the existence of molecules that are important for this enhancement of memory extinction due to a previous quick retrieval session, been GluA1 one of this molecules. Almeida-Correa and Amaral (2014) refers to the observations of Clem and Huganir (2010) that blocking of GluA1 phosphorylation at serine 845, by genetic

mutation, impairs enhancement of memory extinction by a previous retrieval session. This shows that GluA1 is an important molecule for the interaction between memory reconsolidation and memory extinction.

The separation between this two possible interpretations for our results is an important next step, however it will be time consuming, requiring many different experiments, and escapes the aims set for this thesis. To separate between a destabilization impairment and memory extinction enhancement more molecular data (as for example expression of BDNF and ERK) and behavioural data (test for reinstatement of memory and spontaneous recovery) are necessary. Even in the possession of new data we are still not certain to be able to separate this two hypothesis, since they might be different interpretations of the same phenomenon (Almeida-Correa and Amaral, 2014; Trent et al., 2015).

Independent of the hypothesis used to explain the retrieval-induced memory impairment observed after hippocampal injection of shCaMK2N1 virus solution, the phenotype observed (Figure 3.3-2) still implicates in a clinical potential of CaMK2N1 knockdown in the treatment of PTSD and addiction. PTSD, as defined in the DSM V (Association, 2013), is an axis I anxiety disorder that develops as a result of experiencing intense stressor, usually involving threat of serious injury or death to oneself or to someone nearby. Patients with PTSD have been observed to have a decrease in hippocampal volume (Bremner et al., 1995; Gurvits et al., 1996; Stein et al., 1997). Memory impairments have also been reported in PTSD patients (Bremner et al., 1993; Horner and Hamner, 2002; Vasterling et al., 1998). Based on experiments with twin patients, Gilbertson et al. (2007) and Gilbertson et al. (2002) have proposed that pre-existing abnormalities in the hippocampus might facilitate the prevalence of PTSD.

Moreover, PTSD patients have been observed to maintain the conditioned fear responses to the traumatic stimuli even 40–50 years after the event (Orr et al., 1993). Different molecules have been tested as therapeutic targets to pharmacologically erase or block the traumatic memory (Fitzgerald et al., 2014; Pizzimenti and Lattal, 2015). Based on the retrieval-dependent fear memory impairment observed on this thesis (Figure 3.3-2) we proposed the inhibitors of CaMKII to be a possible therapeutic target for PTSD. Exposure of animals to a single prolonged stress (SPS), a paradigm used as animals model of PTSD, has been shown to decrease hippocampal levels of αCaMKII one day after SPS exposure (Han et al., 2013; Liu et al., 2013). A decrease in prefrontal cortex αCaMKII levels was also observed 1 day after SPS (Wen et al., 2012). Similar results were also reported for the basolateral amygdala (Xiao et al., 2009). Moreover, intraperitoneal injection of the histone deacetylase inhibitor vorinostat facilitates CFC memory extinction and increases hippocampal αCaMKII and βCaMKII levels in SPS exposed animals (Matsumoto et al., 2013). Thus, testing the effects of hippocampal inhibition of CaMK2N1 in PTSD animal models could help understand the diseases and find molecular pathways for treating PTSD.

The same principle could be applied for addicted patients. Drug seeking behaviour could be interpreted as a CR to the stimulus of the drug. In other words, the associative memory between the pleasure sensation of the drug and the intake of it, or the association of the displeasure of the withdrawal and not taking the drug, are strong memories that result in craving and increase drug intake even long into abstinence (Everitt, 2014). Treatments able to block or erase these memories might be an effective way of treating addicted patients (Everitt, 2014; Pizzimenti and Lattal, 2015). Therefore, it is likely that CaMK2N1 knockdown might decrease the occurrence of drug seeking

behaviour. To my knowledge the knockdown of any CaMKII endogenous inhibitor has never been tried as a treatment for addiction. It is known though that CaMKII expression is increased in the nucleus accumbens (NAc) after chronic cocaine (Robison et al., 2013) and amphetamine (Loweth et al., 2010) intake. It has also been shown that pharmacological blocking of CaMKII activity in the NAc reduces locomotor response to cocaine (Pierce et al., 1998) and amphetamine (Loweth et al., 2008). Additionally, overexpression of αCaMKII in the NAc, by viral transfection, increases amphetamine self-administration (Loweth et al., 2010). Not much is known about the function of hippocampal CaMKII in cocaine and amphetamine addiction. Recent publication by Schopf et al. (2015) showed that cocaine and alcohol treatment induced an increase in c-fos expression in the DG that was blocked by T286A mutation.

Regarding alcohol, it has been observed that T286A mutants have a decrease in initial alcohol intake and decrease in locomotor response for low doses of alcohol (2 g/Kg) (Easton et al., 2013b). Furthermore, T286A mutants also present an increase in c-fos expression in the rostral region of the ventral tegmental area (Easton et al., 2013b). T268A mutants also have a facilitation in learning of conditioned place preference using alcohol as reinforcement (Easton et al., 2013c). Moreover, increase in locomotor activity induced by the test context was abolished by alcohol in T286A mutants, but not in control mice (Easton et al., 2013c). Easton et al. (2013b) also reported a significant correlation between alcohol dependence in humans and a single nucleotide polymorphism within an autophosphorylation-related area of αCaMKII gene. Considering the relevance of CaMKII and its autophosphorylation in alcohol, amphetamine and cocaine intake and addiction, it is probable that the inhibitors of CaMKII have a function in addiction. Experiments with shCaMK2N1 and CaMK2N2 virus

solutions could help understand the function of CaMKII inhibitors in the effects of cocaine, alcohol and amphetamine addiction.

5.2 CaMK2N2 overexpression

CaMK2N2 hippocampal overexpression, induced by treatment with CaMK2N2 virus solution, resulted in a learning impairment of CFC task (Figure 4.2 1). This result is in accordance with Buard et al. (2010) observations that treatment with tatCN21 inhibits LTP induction and impairs learning of CFC task. We also observed that hippocampal CaMK2N2 overexpression after learning of the CFC task did not affect memory maintenance (Figure 4.3-2). The same results were also obtained by Buard et al. (2010) using intraperitoneal injection of tatCN21. This indicates that CaMKII activation is necessary for memory formation but not memory maintenance. However, is important to notice that this does not mean that CaMKII is not relevant for memory maintenance. As presented in the last section of this discussion (5.1 CaMK2N1 knockdown), it seems that although activation of CaMKII is not important for CFC memory maintenance its inactivation is. It is important to consider as well that CaMKII has been suggested to also have a structural function relevant to memory maintenance. CaMKII binding to elements of the cytoskeleton could be relevant for changes in synapse morphology that may have a direct impact in memory maintenance (Fink et al., 2003; Jiao et al., 2011; Okamoto et al., 2009; Robison et al., 2005b). CaMKII may also act as a scaffolding protein, binding to NMDAR at the PSD and regulating the numbers of AMPAR at the synapses, which could also be important for memory maintenance (Sanhueza and Lisman, 2013). In this regard, the CaMK2N2 virus solution used in this thesis can be used as an efficient and selective way of studying CaMKII structural roles. It is known that the endogenous inhibitors of CaMKII block binding to NMDAR (Vest et al., 2007). Therefore various experiments can

be propoused to link memory maitaince to the molecular mechanisms suggested. For example, Sanhueza and Lisman (2013) theory could be tested by using co-imunoprecptation analysis to check if CaMK2N2 overexpression after CFC training has any effect on CaMKII binding to AMPAR. Since CaMK2N2 hippocampal overexpression had no effect on memory maitainance, based on Sanhueza and Lisman (2013) hypothesis it should also not have affeted CaMKII and AMPAR co-preciptation.

The lack of effect of CaMK2N2 overexpression in memory maintenance (Figure 4.3-2) could also be explained by the longer duration between training and memory test (15 days as opposed to 24 hours). A more classical interpretation of the function of the hippocampus in memory is that memories would only persist in the hippocampus for a short period of time, “migrating” later to other regions (Anagnostaras et al., 1999; Kim and Fanselow, 1992). If that was the case, it implies that the lack of effect of our treatment would be a result of it been focus on the hippocampus, a region that would no longer be involved in the maintenance of the memory at a later time point. However, Buard et al. (2010) treated animals with intraperitoneal injection of tatCN21 and also observed no effect in memory maintenance. Furthermore, the classical transient role of the hippocampus in memory has been challenged by the report of an amnesic effect of optogenetic-induced hippocampal inactivation even for older memories (Goshen et al., 2011). As proposed by Goshen et al. (2011), the hippocampus seems to be important for the maintenance of older memories, however if lesioned or inactivated for a prolonged period of time other regions of the brain could replace his function.

By comparing the behavioural results obtained for CaMK2N2 overexpression before (Figure 4.2-1) and after training (Figure 4.3-2) there is a noticeable difference in the freezing scores obtained during the two experiments. When testing CaMK2N2

overexpression before training, control animals froze approximately 30% of the time in the first memory test. On the other hand, when testing the effects of CaMK2N2 overexpression after training control animals froze approximately 70% in the first memory test. This difference in freezing scores is not related to CaMK2N2 overexpression since the effect is present in control animals. Therefore, the difference duration between training and memory testing might cause distinct freezing levels in the memory test (24 hours in the first experiment and 15 days in the second). Indeed it has been shown that a longer period of separation between the memory formation and memory test increases the occurrence of the CR during the test, a phenomenon that was called memory incubation (Brady, 1951; Eysenck, 1968; Houston et al., 1999).

A closer look at these two behavioural results (Figure 4.2-1 and Figure 4.3-2) also brings to the attention the occurrence of an effect of the memory tests in both experiments. Once again, this effect was not related to CaMK2N2 expression since it was also present in the control group and no significant effect of interaction was observed. These results seem to indicate that both, animals from Control virus group and from CaMK2N2 virus group, had a small extinction of the memory from the first to the second memory test. During the performance of these two experiments a new building was been constructed next door to our animals facilities unit. It is possible that the noise of the construction may have caused some level of stress to the animals. This stress might be responsible for inducing memory extinction in the animals. In fact, injection of corticosterone, a stress-induced molecule, has been show to facilitate extinction of CFC memory (Cai et al., 2006). Nonetheless, the results obtained in our experiments are in accordance with previous data published by Buard et al. (2010) and both groups (Control virus and CaMK2N2 virus) were exposed to the same conditions. Thus, any possible

stress-induced effect on memory did not have any relevant impact in the conclusions draw from our experiments.

Mutation R495X in the FUS gene (also known as FUS-ΔNLS), an ALS causing mutation (Renton et al., 2014), has been shown to induce an overexpression of CaMK2N2, *in vitro* (Convertini et al., 2013). Furthermore, TAR DNA binding protein-43 (TDP-43) mutated animals, an animal model for ALS and frontotemporal lobar degeneration (FTLD) (Barmada and Finkbeiner, 2010; Janssens and Van Broeckhoven, 2013), has also been observed to present an increase in CaMK2N2 expression (unpublished data from Dr. Shou-Chien Ling at National University of Singapore). TDP-43 accumulation with RNA molecules forming inclusions inside neurons, has been shown to occur in ALS and FTLD (Arai et al., 2006; Bentmann et al., 2013; Neumann et al., 2006; Wolozin, 2012). Wang et al. (2008) has reported the mRNA of αCaMKII to be among the mRNAs trapped in TDP-43 inclusions. Additionally, mutation inducing loss of function in progranulin protein, a FTLD animal model known to induce the appearance of TDP-43 inclusions, has been show to induce increase in CaMKII T286 phosphorylation (Alquezar et al., 2014). Therefore, it is likely that the inhibitors of CaMKII, specifically CaMK2N2, play a role in ALS and FTLD. Experiments with CAMK2N2 virus solution as a possible model for some of the symptoms of these diseases would be an important next step. ALS is a neurodegenerative disorder characterized by selective death of motor neurons in the brain and spinal cord. The initial symptoms are paralysis of voluntary muscles followed, rapidly, by death from respiratory failure, with a typical onset in adult life (Mulder, 1982; Rowland and Shneider, 2001). FTLD is described as neurodegenerative diese characterized by the relatively selective death of neurons in the anterior and frontal temporal lobes. However, FTLD is in reality a group of different disorders that

are related due to this prevalence of imapriment in the anterior and frontal temporal lobes. FTLD is a presenile dementia, with onset usually before 65 years old (Josephs, 2008; Josephs et al., 2011; Neary et al., 2005; Rabinovici and Miller, 2010; Whitwell and Josephs, 2011). ALS and FTLD are multifactorial neurodegenerative diseases (Josephs et al., 2011; Ling et al., 2013; Pasinelli and Brown, 2006; Renton et al., 2014; Sieben et al., 2012), and have been proposed to be part of a continuum spectrum of the same biological pathology (Geser et al., 2009; Ling et al., 2013). This hypothesis is corroborated by existence of motor neuron dysfunction in FTLD patients (Burrell et al., 2011) and impaired performance in frontal lobe tests in ALS patients (Ringholz et al., 2005). No efficient treatment for these diseases are known (Janssens and Van Broeckhoven, 2013; Ludolph et al., 2007; Whitwell and Josephs, 2011). Studies to understand CaMK2N2's role in ALS and FTLD might help revealing new therapeutic targets for the treatment of these diseases.

5.3 CaMK2N1 and CaMK2N2

During the first behavioural experiment with shCaMK2N1 virus solution it was observed to cause an effective CaMK2N1 knockdown (Figure 3.3-3) but it also increased CaMK2N2 expression (Figure 3.3-4). This increase could be interpreted as plastic compensation of the brain to our virus treatment. In order to exclude the possibility of the behavioural phenotype observed after hippocampal CaMK2N1 knockdown (Figure 3.3-2) been an effect of the compensatory increase in CaMK2N2 expression, we modelled the increase of CaMK2N2 observed, using a 60X diluted version of our CaMK2N2 virus solution (Figure 4.4-2). Our results seem to indicate that an increase of up to approximately 3X in CaMK2N2 mRNA levels in the dorsal hippocampus (Figure 4.4-4) does not have any effect on CFC learning and memory (Figure 4.4-3). Therefore, it is unlikely that the retrieval-induced memory impairment observed after CaMK2N1 knockdown (Figure 3.3-2) is induced by the CaMK2N2 increase (Figure 3.3-4) also observed.

This suggests that although the animals' brain tried to compensate the virus-induced decrease in CaMK2N1 with an increase in CaMK2N2 expression, it was not enough to replace CaMK2N1 function. Hence, is likely that CaMK2N1 and CaMK2N2 have different roles in memory maintenance. This hypothesis is further corroborated by the observation that different intensities of the US, during training session, resulted in different regulation, in the dorsal hippocampus, of CaMK2N1 (Figure 3.5-2 and Figure 3.5-5) and CaMK2N2 (Figure 3.5-3 and Figure 3.5-6) expression after memory retrieval. Considering that CaMK2N2 requires constant presence of CaM to ligate to CaMKII (Chang et al., 2001) a stronger US (5 shocks) might generate a stronger and/or longer calcium influx and CaM availability in the neuron, which justify the increase in CaMK2N2

expression. CaMK2N1, on the other hand, might be expressed when the stimulus produces a smaller influx of calcium and less CaM avaiblity, since this inhibitor does not require CaM to ligate to CaMKII (Chang et al., 2001) and is expressed after weaker US presentation (3 shocks). It has been demonstrated in this thesis that CaMK2N1 has a function in regulating CaMKII autophosphorylation (Figure 3.6-3), AMPAR synaptosomal levels (Figure 3.6-6), and gene expression (Figure 3.6-19) after CFC memory retrieval. CaMK2N1 seems to inhibit synapse activation and consequent gene expression in order to maintain CFC memory after retrieval (Figure 5.1-1).

CaMK2N2's function in memory maintenance is still to be determined. The knockdown of CaMK2N2 by viral vector injection in the hippocampus, after and before 5 shocks CFC task, might be a good experiment to help understand CaMK2N2 function in memory maintenance. During the executeon of this thesis we have tried to knockdown CaMK2N2 expression, in the hippocampus, with viral vector treatment. However, *in vivo* experiments showed that the treatment had no effect on CaMK2N2 expression (Appendix Figure 7.6-1). The construct of more viral vectors, with shRNA sequences that target different regions of CaMK2N2 mRNA, is required. We have also attempted to overexpress CaMK2N1 by viral vector treatment and failed (Appendix Figure 7.7-1). Possession of 4 viral vectors, to overexpress and knockdown both endogenous inhibitors of CaMKII, would provide a good tool to test overlapping functions between CaMK2N1 and CaMK2N2. Experiments knocking down one inhibitor and overexpressing the other could access the ability of one inhibitor in compensate for the function of the other. It would also be relevant to test the effects of knocking down both inhibitors in CFC learning and memory maintenance.

Another possible approach for studying CaMK2N1 and CaMK2N2 functions is the generation of mice with mutations in the genes encoding the inhibitors. Nonetheless, the use of mutant animals for studying the functions of any molecule brings the caveat of causing alterations in every cell that this molecule is expressed and it might as well cause development disorders. For example, βCaMKII knockout mice have motor impairments that restrict the number of possible cognitive tests that can be used in these animals, as they also present a developmental delay in body weight that could interfere with behavioural studies at earlier stages of development (Bachstetter et al., 2014; Borgesius et al., 2011; van Woerden et al., 2009). Additionally, CaMKII has been shown to play a role in neuronal proliferation and neurite growth, which implies that mutations in CaMK2N1 or CaMK2N2 genes may possibly result in brain development problems. Saito et al. (2013) has observed that knockdown of βCaMKII, but not αCaMKII, suppressed neuritogenesis, while βCaMKII activation increases neuritogenesis. This βCaMKII effect on neuritogenesis was related to the phosphorylation of LIM-kinase 1, at threonine 508, by βCaMKII, promoting the activation of LIM-kinase 1 (Saito et al., 2013). It has also been reported that mice heterozygous for a null mutation of αCaMKII have an increase in bromodeoxyuridine (BrdU) incorporation in DG cells but a decrease in the number of mature neurons in the same hippocampal area, as well as alterations in the expression of more than 2000 genes (Yamasaki et al., 2008). One should also consider that CaMK2N2 is expressed in testis (Chang et al., 2001), where its function is still unknown. Mutations in CaMK2N2 gene may have deleterious consequence in hormonal production in the testis that could cause behavioural and development alterations.

Viral vectors like the ones used in this thesis, on the other hand, have the advantage of allowing manipulation of gene expression in a specific region and at a time

point desired, avoiding side effects in other areas and in development. In fact, the use of viral vectors has been suggested to be an efficient tool to study mechanisms underlying neuropsychiatric disorders as well as to be possibly useful for treatment of such disorders (Edry et al., 2011; Ginn et al., 2013; Murlidharan et al., 2014; Ojala et al., 2015). The use of AAV vectors has received particular attention due to their safety profile (very low chance of insertion-induced mutations) and efficiency in transducing a wide range of cell types (Ginn et al., 2013; Murlidharan et al., 2014; Ojala et al., 2015). Indeed, recent successful clinical trials with AAV vectors have been reported for haemophilia B and the retinal degenerative disorder Leber's congenital amaurosis type 2 (Bainbridge et al., 2008; Cideciyan et al., 2009; Nathwani et al., 2011; Testa et al., 2013). In terms of neuropsychiatric disorders, clinical trials with Alzheimer's disease (Mandel, 2010) and Parkinson's disease (Bartus et al., 2013; LeWitt et al., 2011) are currently been held. A big challenge in the area is imposed by the blood brain barrier ability to block the access of the viral vectors to the central nervous system. For this reason clinical trials have so far been using intracranial or intracerebrospinal fluid injections of the vectors. Attempts with intravenous injection of AVV viral vectors have been made, but side effects in other tissues have been reported (Gray et al., 2011), making further studies necessary. More studies in the tropism of different types of AVV for specific cells are also required. AVV type 2, the one used in this thesis, has been shown to have a strong tropism for neuronal cells (Bartlett et al., 1998; Davidson et al., 2000) and is a good candidate for treatment of neuropsychiatric disorders. The relevance of CaMKII inhibitors in different neuropsychiatric disorders as well as the use of our viral vectors solutions in animal models of these diseases are discussed in detail at session "5.4 CaMKII endogenous inhibitors as therapeutic target for neuropsychiatric diseases".

The study of the function of CaMKII endogenous inhibitors in other brain regions is also an interesting future experiment. The amygdala, for example, would be an area of much interest. The amygdala has been shown to play a key role in fear conditioning, with lesion of this region causing impairment in both cued and context fear conditioning (Phillips and LeDoux, 1992). Furthermore, injection of anisomycin in the amygdala has a retrieval-induced amnesic effect (Nader et al., 2000) and increase in c-fos expression has been shown to occur in the amygdala after memory retrieval (Do-Monte et al., 2015). The amygdala has also been shown to participate in memory destabilization, reconsolidation and extinction (Kim et al., 2014; Mamiya et al., 2009; Milton et al., 2013). Considering this relevance of amygdala in different memory retrieval-induced processes and the fact that the endogenous inhibitors of CaMKII are expressed in this area of the brain (Chang et al., 2001; Chang et al., 1998; Radwanska et al., 2010), the amygdala becomes an interesting candidate for further experiments with our viral vector solutions.

It would also be interesting to study the effects of changes in CaMK2N1 and CaMK2N2 expression on different neurotransmitters, besides the glutamatergic system. CaMKII has been show to regulate and/or been regulated by serotonin (Asaoka et al., 2015; Ciccone et al., 2008; Moyano et al., 2004; Steinkellner et al., 2015) and GABA (Churn and DeLorenzo, 1998; Ghosh et al., 2015; Marsden et al., 2010; Saliba et al., 2012; Zhang et al., 2004), making these two neurotransmitters interesting candidates for more studies on the function of CaMKII endogenous inhibitors.

Experiments with calcium channel and the inhibitors of CaMKII are also recommended. It is known that CaMK2N1 blocks binding of CaMKII to the calcium channel $Ca_V2.1$ (Magupalli et al., 2013). However it is unknown if CaMK2N2 has the same

ability and if binding to other calcium channels area also affected by the endogenous inhibitors of CaMKII. Experiments with CaMK2N2 overexpression might help understand the answer to these questions. The calcium channel $Ca_v1.2$ is known to bind and be phosphorylated by CaMKII, which seems to increase $Ca_v1.2$ levels (Hudmon et al., 2005; Lee et al., 2006; Simms et al., 2015). Therefore is likely that the endogenous inhibitors of CaMKII regulate CaMKII interaction with $Ca_v1.2$, but this has not been tested experimentally.

In this thesis we have not assessed any effects on neurogenesis by the two virus treatments. The DG is known to be one of the two brain areas where new neurons are born throughout adulthood, a process considered to be important for cognition (Lazarov et al., 2010; Shors, 2004). Ji et al. (2014) observed that forcing rats to run resulted in increase in DG neurogenesis and increase in T286 αCaMKII phosphorylation, and combining forced run with whole-brain irradiation reverse the decreased in neurogenesis caused by the irradiation as well as increases T286 αCaMKII phosphorylation. Furthermore, treatment of cultured DG cells with methamphetamine decreases cell proliferation and total CaMKII levels. Combining methamphetamine treatment with melatonin treatment attenuated the effects of methamphetamine in cell proliferation and CaMKII levels (Ekthuwapranee et al., 2015). Thakker-Varia et al. (2014) reported that treatment of cultured hippocampal neurons with the peptide TLQP-62 increase neuronal proliferation and T286 αCaMKII phosphorylation, while blocking of CaMKII by KN-93 blocks the effects of TLQP-62 on neuronal proliferation. Therefore, is likely that CaMKII plays an important role in neurogenesis and study the effects of our vector virus treatments in neurogenesis could be an interesting future experiment.

5.4 CaMKII endogenous inhibitors as therapeutic target for neuropsychiatric diseases

Although only ALS (Convertini et al., 2013) and FTLD (unpublished data from Dr. Shou-Chien Ling at National University of Singapore) have been directed linked with the inhibitors of CaMKII, the study of the functions of CaMK2N1 and CaMKN2 might increase knowledge in the field of other dieses that have been related to CaMKII. We have proposed in this thesis a role for CaMK2N1 in PTSD and addiction treatment. CaMKII has also been suggested to play an important role in depression (Li et al., 2013; Robison, 2014). Li et al. (2013) observed that βCaMKII expression was increased in the lateral habenula of different animal models of depression, and that knockdown of βCaMKII expression in this region reduced depression-like behaviours. Therefore, an interest experiment would be to test if the injection of our CaMK2N2 virus solution in the lateral habenula of animal models of depression decrease depression-like behaviours. Additionally, the effect of this treatment could be compared to the effect of intraperitoneal injection of tatCN21.

Another disease that has been related to CaMKII is Alzheimer's disease (AD). First described by Dr. Alois Alzheimer in 1906, AD is the most common cause of dementia and is identified by accumulation of beta amyloid (Aβ) plaques, tangles of hyperphosphorylated tau protein, and decrease in brain volume. All of those hallmarks of AD seem to occur in specific areas of the brain with the hippocampus been one of the first to be affected. Furthermore, loss of spatial cognition is one of the first symptoms of AD (Agrawal and Biswas, 2015; Fellgiebel and Yakushev, 2011; Hardy and Selkoe, 2002; Jucker and Walker, 2013; Lage, 2006; Rachakonda et al., 2004; Wang, 2014). Reese et al. (2011) have observed that AD patients and patients with mild cognitive impairment

have an altered distribution of T286 phosphorylated αCaMKII from dendritic arborisations to neural perikarya in the hippocampus. This change in distribution of αCaMKII correlates with scores in cognitive tests. Additionally, Reese et al. (2011) also reported that intracerebroventricular injection of Aβ oligomers in mice resulted in the same alterations in T286 phosphorylated αCaMKII localization and treatment of primary hippocampal neurons with the same Aβ solution caused a reduction in synaptic spines immunostaining. Levels of T286 phosphorylated αCaMKII have also been shown to be reduced in the hippocampus of animal models of AD and vascular dementia (Min et al., 2013). Same AD animal model also presents a decrease in $Ca_v1.2$ levels and decrease colocalization of CaMKII and $Ca_v1.2$ in the hippocampus (Min et al., 2013). Using a rat model with hyperhomocysteinemia, a risk factor of AD, Jiang et al. (2015) reported that training in MWM task improves animals' performance in the CFC task and also increases hippocampal GluA1 levels and DG dendritic ramification and spine generation. Moreover, injection of KN93 into the DG blocked the training-induced cognitive improvement, spine generation and dendritic ramification, indicating that DG CaMKII is necessary for these MWM training-induced effects (Jiang et al., 2015). It is important to remember that loss synapses has the strongest correlation with the cognitive decline of AD patients ever observed (DeKosky and Scheff, 1990; Terry et al., 1991). The observations of Jiang et al. (2015), Min et al. (2013) and Reese et al. (2011) have stablished a direct link between AD and CaMKII, therefore it is possible that the endogenous inhibitors of CaMKII may also play a role in the progression of this dieses. Interestingly, Ohno (2009) has observed a retrieval-induced memory impairment in mutant animals with five AD-related mutations when animals were tested for the second time in the CFC paradigm, much like our observation after CaMK2N1 knockdown (Figure 3.3-2).

Changes in CaMKII autophosphorylation has been linked with α-thalassemia X-linked mental retardation (ATRX). ATRX is a syndrome characterized by alterations in craniofacial features, genitalia, hypotonia, mild-to-moderate anaemia, intellectual disability and developmental delay (Stevenson, 1993). In an ATRX animal model Shioda et al. (2011) observed an increase in T286 and T287 CaMKII autophosphorylation and elevated CaMKII autonomous activity in the medial prefrontal cortex. This increased autophosphorylation seems to be due to decreased levels of PP1, a known phosphatase of CaMKII (Blitzer et al., 1998; Brown et al., 2000; Strack et al., 1997), and the increase in kinase activity was specific to CaMKII as the activities of CaMKI, CaMKIV, ERK and PKCα are not changed in the ATRX mouse model (Shioda et al., 2011). On the other hand, the hippocampus of the same mouse model has decreased αCaMKII T286 autophosphorylation (Nogami et al., 2011). These observations suggest that the endogenous inhibitors of CaMKII are likely to have a role in ATRX.

Juvenile male stroke-prone spontaneously hypertensive rats, an animal model of attention-deficit/hyperactivity disorder (ADHD), has also shown T286 and T287 CaMKII autophosphorylation increase in prefrontal cortex. In this ADHD model GluA1 phosphorylation at the S831 was also elevated. Furthermore, those animals have impaired performance in in the Y-maze and novel object recognition tasks. Interestingly, acute treatment with methylphenidate reverts the behavioural phenotypes and restores T286/287 and GluA1 phosphorylation to normal levels (Yabuki et al., 2014). Experiments with this ADHD model and our virus might also increase knowledge of disease-causing mechanisms. ADHD is a disorder that is difficult to diagnose. As determined in DSM V (Association, 2013) children must have at least six symptoms from the two groups of symptoms, been those an inattention group of criteria and the

hyperactivity and impulsivity criteria. Adolescents and adults, on the other hand, must have at least five symptoms to reach criteria. Symptoms are various, including for example trouble holding attention, excessive talking, trouble waiting, difficulties in performing tasks that require prolonged mental effort, trouble organizing tasks and activities and many others (Association, 2013).

CaMKII has also been suggested to play role in schizophrenia (Frankland et al., 2008; Robison, 2014). To diagnose schizophrenia, as defined in the DSM V, the patient needs to present two or more of the following symptoms, been mandatory the presentation of one of the first 3 symptoms: Delusions; Hallucinations; Disorganized speech; Grossly disorganized or catatonic behaviour; Negative symptoms (for example diminished emotional expression). Frankland et al. (2008) and Robison (2014) suggestion of a possible role of CaMKII in schizophrenia is centred on the observations by Yamasaki et al. (2008) that heterozygous mice for a null mutation in αCaMKII have behavioural abnormalities that are also found in patients with schizophrenia, like decrease sociability, infradian rhythm (daily cycle of more than 24 hours) and working memory deficits. Additionally, Yamasaki et al. (2008) also reported that gene expression alterations in the DG of these animals are clustered together with known genetic alterations of schizophrenic patients. A possible role for the endogenous inhibitors of CaMKII has never been addressed and could increase our knowledge of this complex disease.

Another disease that has been related to CaMKII alterations is Angelman syndrome (AS). AS is a neurodevelopment disorder that occurs due to a maternally inherited dysfunction in the ubiquitin ligase E3A. AS symptoms include seizures, motor impairment, cognitive disability and speech disorders (Sell and Margolis, 2015; Thibert

et al., 2013). Animals models of AS, with dysfunctional ubiquitin ligase E3A, have a reduction in BDNF-induced T286/287 CaMKII phosphorylation in the hippocampus and cortex, but increase basal levels of T286/287 phosphorylation in the same areas (Cao et al., 2013). Weeber et al. (2003) observed, in the same animal model of AS, an increase in hippocampal levels of T286 αCaMKII phosphorylation but also an increase in T305 phosphorylation and a reduction in CaMKII kinase activity, autophosphorylation capability, and CaMKII levels at the PSD. Steinkellner et al. (2012) reported that animals with phosphorylation-mimicking mutation of the 305 site of αCaMKII (T305D) have a similar impairment in dopamine transport as the one observed in the AS animal model. Additionally, AS animal model have memory deficits in the MWM and CFC, but this deficits can be reversed by the introduction of a mutation on the T305/306 site of CaMKII, blocking its phosphorylation (van Woerden et al., 2007). Considering that the endogenous inhibitors of CaMKII are known to block T305 CaMKII phosphorylation (Vest et al., 2007) we suggested that changes in CaMK2N1 and CaMK2N2 expression might have a potential therapeutic effect on AS.

Epilepsy is a neurologic disorder characterised by excessive neuronal firing due to an imbalance in glutamatergic versus GABAergic neurotransmission, resulting in seizures (DiNuzzo et al., 2014; During and Spencer, 1993). CaMKII has also been proposed to play a role in epilepsy (Liu and Murray, 2012; Robison, 2014). Different epileptic-inducing treatments have been reported to result in a decrease in T286 phosphorylation. Such treatments include administration of GABA-receptor antagonists (Bronstein et al., 1988a; Perlin et al., 1992), pilocarpine-induced seizures (Churn et al., 2000a; Kochan et al., 2000), kainic acid treatment (Yamagata et al., 2006), and in kindled rats (Goldenring et al., 1986; Wasterlain and Farber, 1984; Wu et al., 1990).

Corroborating with a possible role of CaMKII in epilepsy, Butler et al. (1995) observed that αCaMKII knockout results in facilitation of epileptic seizure by electric stimulation due to hyperexcitability of limbic structures, including the hippocampus. Moreover, in cultured hippocampal neurons with inhibition of αCaMKII expression (Churn et al., 2000b) or in cultured cortical neurons subjected to tatCN21 treatment (Ashpole et al., 2012) epileptic-like activity was observed. Considering that decrease in CaMKII phosphorylation, expression and activity has been directly linked to epilepsy it is possible that inhibition of CaMK2N1 by shCaMK2N1 virus solution might have positive effects in the treatment of epilepsy. More experiments are necessary to confirm this hypothesis.

The use of inhibitors of CaMKII in the treatment of cardiological diseases, cancer and rheumatoid arthritis has also been suggested (Pellicena and Schulman, 2014; Wang et al., 2015c). Table 5.4-1 summarize the neuropsychiatric disorders that have been presented in this thesis due to their association with either CaMKII or CaMKII endogenous inhibitors.

Diseases	Reference
Amyotrophic lateral sclerosis	Convertini et al. (2013) Wang et al. (2008)
Frontotemporal lobar degeneration	Wang et al. (2008) Alquezar et al. (2014) Unpublished data from Dr. Shou-Chien Ling at National University of Singapore
Addiction	Robison et al. (2013) Loweth et al. (2010) Loweth et al. (2010) Pierce et al. (1998) Schopf et al. (2015) Easton et al. (2013b) Easton et al. (2013c) Easton et al. (2013a)
Post-traumatic stress disorder	Han et al. (2013) Liu et al. (2013) Wen et al. (2012) Xiao et al. (2009) Matsumoto et al. (2013)
Alzheimer's diseases	Jiang et al. (2015) Min et al. (2013) Reese et al. (2011)
Depression	Li et al. (2013)

α-thalassemia X-linked mental retardation	Nogami et al. (2011) Shioda et al. (2011)
Attention-deficit/hyperactivity disorder	Yabuki et al. (2014)
Schizophrenia	Yamasaki et al. (2008)
Angelman syndrome	Cao et al. (2013) Weeber et al. (2003) Steinkellner et al. (2012) van Woerden et al. (2007)
Epilepsy	Bronstein et al. (1988a) Perlin et al. (1992) Churn et al. (2000a) Kochan et al. (2000) Yamagata et al. (2006) Wasterlain and Farber (1984) Goldenring et al. (1986) Wu et al. (1990) Butler et al. (1995) Churn et al. (2000b) Ashpole et al. (2012)

Table 5.4-1 Neuropsychiatric disorders related to CaMKII

This table presents the psychiatric disorders that have been presented in this thesis due to their known association with CaMKII or with CaMKII endogenous inhibitors. The first column shows the name of the diseases and the second column show the reference that associates it with CaMKII. It is my believe that experiments manipulating CaMK2N1 and/or CaMK2N2 expression in animal models of these diseases might help increase knowledge of the molecular mechanisms of these disorders and find novel therapeutic targets for them.

Chapter 6 Conclusion

In conclusion, we have shown for the first time that CaMK2N1 is necessary for memory maintenance after retrieval. We have also shown for the first time that T286 phosphorylation of CaMKII is decreased 2h after retrieval of 1-shock-induced contextual fear memory. The data presented in this thesis seems to suggest that CaMK2N1 is expressed after memory retrieval and that this expression is necessary to block CaMKII activation, decrease AMPAR levels in the synapses and c-fos nuclear expression in order to keep the memory.

We have also confirmed that CaMKII activation is necessary for memory formation but not for memory maintenance in agreement with Buard et al. (2010) observations.

Therefore this thesis has presented valid new data for the field of molecular bases of learning and memory. Our data, allied with different observations reported in the literature, suggests the endogenous inhibitors of CaMKII as potential therapeutic target for various diseases.

Chapter 7 Appendix

7.1 Sequence information of plasmids

Name	Sequence (5' – 3')
Scrambled shRNA	GGATCCTCGCTTGGGCGAGAGTAAGTTCAAGAGACTTACTCTCGCCCAAGCGATTTTTTACTAGTGGATCC
ShCaMK2N1	GGATCCGAGCAAGCGCGTTGTTATTCTCAAGAGAAATAACAACGCGCTTGCTCTTTTTTACTAGTGGATCC
CaMK2N2	GAATTCACCATGTCCGAGATCCTACCCTACGGTGAGGACAAGATGGGCCGCTTCGGCGCAGACCCCGAA GGTTCCGACCTCTCTTTCAGCTGCCGCCTGCAGGACACCAACTCCTTCTTCGCTGGCAACCAGGCCAAGCG GCCCCCCAAGCTGGGCCAGATCGGCCGAGCCAAGAGAGTGGTGATCGAGGATGACCGGATAGACGACG TGCTGAAGGGGATGGGGGAGAAGCCTCCGTCCGGAGTGTAG
Ampicillin resistance	AAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGT TTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTA CATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATG AGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTC GCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGG CATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTG ACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTT GATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCA ATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAG ACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGC TGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCC CTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGC TGAGATAGGTGCCTCACTGATTAAGCATTGGTAA
5' ITR	CTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCG GCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCCT
3' ITR	AGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACC AAAGGTCGCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAG
Synapsin 1 promoter	GAGGGCCCTGCGTATGAGTGCAAGTGGGTTTTAGGACCAGGATGAGGCGGGGTGGGGGTGCCTACCTG ACGACCGACCCCGACCCACTGGACAAGCACCCAACCCCCATTCCCCAAATTGCGCATCCCCTATCAGAGA GGGGGAGGGGAAACAGGATGCGGCGAGGCGCGTGCGCACTGCCAGCTTCAGCACCGCGGACAGTGCC TTCGCCCCCGCCTGGCGGCGCGCGCCACCGCCGCCTCAGCACTGAAGGCGCGCTGACGTCACTCGCCGG TCCCCCGCAAACTCCCCTTCCCGGCCACCTTGGTCGCGTCCGCGCCGCCGCCGGCCCAGCCGGACCGCAC CACGCGAGGCGCGAGATAGGGGGGCACGGGCGCGACCATCTGCGCTGCGGCGCCGGCGACTCAGCGCT GCCTCAGTCTGCGGTGGGCAGCGGAGGAGTCGTGTCGTGCCTGAGAGCGCAGTC
U6 promoter	TGTAGTTAATGATTAACCCGCCATGCTACTTATCTACGTAGCCATGCTCTAGGAAGATCTGGGCAGGAAG AGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTAGAATT AATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGT TTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCT TGGCTTTATATATCTTGTGGAAAGGACGA

IRES	GCCCCTCTCCCTCCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGTTTGTC TATATGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTG ACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAG CAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCC ACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACC CCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAG GGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCTGGGGCCTCGGTACACATGCTTT ACATGTGTTTAGTCGAGGTTAAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTTGAA AAACACGATGATAATATGGCCACAACC

Table 7.1-1 Sequences inserted in the different plasmids

The sequences presented in this table were inserted in our different plasmids with different proposes. In the column "name" is identified which insert is been address and in the column beside it is the sequence of this insert. Sequences are presented from 5' to 3' direction.

7.2 RT-qPCR primers

Gene	Direction	Sequence (5' – 3')	Concentration
HPRT	Forward	ATACAGGCCAGACTTTGTTGGATT	300nM
	Reverse	TCACTAATGACACAAACGTGATTCAA	300nM
CaMK2N1	Forward	GCTGAAAACCATGACCGACAA	300nM
	Reverse	AAGAATAAGAAAGGGAGTTGTGTAAC	300nM
CaMK2N2	Forward	CGCAGACCCCGAAGGTT	300nM
	Reverse	TTGCCAGCGAAGAAGGAGTT	900nM

Table 7.2-1 Primers' sequence

Table shows the sequence of the primers used in this thesis for RT-qPCR. Both forward and reverse primers are presented for each gene. Sequences are presented from 5' to 3'. Final used concentration for each primer is also presented.

7.3 Solutions & Buffers

7.3.1 Paraformaldehyde solution 4%

Reagent	Volume or Weight
Paraformaldehyde	40g
NaOH (10M) (Sigma-Aldrich, St. Louis, MO, USA; 221465)	≈ 50µL*
10X PBS (Life Technologies, Paisley, UK; 70011-044)	100mL
Water	Complete volume to 1L

Table 7.3-1 Paraformaldehyde solution 4%

This table shows the composition of perfusion solution of PFA 4%. Final pH should be adjusted to 7.5, solution should be filtered and stored in 4°C for no longer than a week. Ultrapure water was used for this solution. *More NaOH (10M) can be added to the solution to facilitate paraformaldehyde dilution.

7.3.2 Reverse transcription solution

Reagent	Volume for 11.5µL of initial solution
10 mM dNTP mix	1µL
0.1M DTT	2µL
RNasin® Inhibitor	0.5µL
SuperScript® II Reverse Transcriptase	1µL
5x first strand buffer	4µL

Table 7.3-2 Reverse transcription solution

For synthesis of the first strand of cDNA the above solution was used. The table describes the volume of each reagent that was added to the initial solution. The initial solution should contain 1.3µg of sample RNA diluted to 10.5µL in nuclease free water and also 1µL of oligo$(dT)_{12\text{-}18}$ primer solution. Initial solution has to be incubated at 70°C for 10 minutes before mixture with the above reverse transcription solution. Reverse transcription solution must be prepared as close to moment of use as possible and kept on ice until used.

7.3.3 RT-qPCR solution

Reagent	Volume for 5µL of cDNA solution
2x qPCR MasterMix	10µL
HPRT forward (5µM)	1.2µL
HPRT reverse (5µM)	1.2µL
CaMK2N1 forward (5µM)	1.2µL
CaMK2N1 reverse (5µM)	1.2µL
CaMK2N2 forward (15µM)	0.4µL
CaMK2N2 reverse (15µM)	1.2µL
Water	Complete volume to 15µL

Table 7.3-3 RT-qPCR solution
Table above describes the volume of each reagent that should be used in RT-qPCR solution. The values presented are relative to one sample of 5µL of cDNA solution. Final reaction solution should contain a total of 20µL been 5µL from the cDNA sample and 15µL of the RT-qPCR solution. RT-qPCR solution must be prepared as close to moment of use as possible and kept on ice until used.

7.3.4 Homogenization buffer for protein extraction

Homogenization buffer*	
Reagent	**Concentration**
Sucrose (Calbiochem® - Merck Millipore, Darmstadt, Germany; 5737)	0.32M
$NaHCO_3$ (Sigma-Aldrich, St. Louis, MO, USA; S6014)	1mM
$MgCl_2$ (Sigma-Aldrich, St. Louis, MO, USA; M2670)	1mM
Hepes (Sigma-Aldrich, St. Louis, MO, USA; H3375)	10mM

For 1mL of the above solution**	
Reagent	**Volume**
Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA; S8820)	15µL
Phosphatase inhibitor cocktail 2 (Sigma-Aldrich, St. Louis, MO, USA; P5726)	10µL
Phosphatase inhibitor cocktail 3 (Sigma-Aldrich, St. Louis, MO, USA; P0044)	10µL

Table 7.3-4 Homogenization buffer for protein extraction

The composition of the homogenization buffer for protein extraction is described on the table above. All reagents should be diluted in ultrapure water. *Final pH of the solution should be 7.4 and stored at 4°C. **Adding of protease and phosphatase inhibitors should be done immediately prior to use.

7.3.5 4X sample buffer for protein electrophoresis

5X sample buffer (Stock solution)*	
Reagent	**Concentration**
Glycerol (Sigma-Aldrich, St. Louis, MO, USA; G5516)	20% v/v
Sodium dodecyl sulphate (SDS) (Sigma-Aldrich, St. Louis, MO, USA; 71727)	10% w/v
Trizma® base (Sigma-Aldrich, St. Louis, MO, USA; 93349)	250mM
Bromophenol blue (Sigma-Aldrich, St. Louis, MO, USA; B0126)	Enough for the solution to get blue

4X sample buffer **	
Reagent	**Volume**
5X sample buffer	80µL

2-Mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA; M6250)	10µL
Water	10µL

Table 7.3-5 4X sample buffer for protein electrophoresis
SDS based sample buffer was used for electrophoresis in this thesis. All solutions were diluted in ultrapure water. *The pH of this solution should be adjusted to 6.7 and it can be stored at RT. **Prior to use the 5X stock solution was diluted to 4X, as described in the table, and this solution was stored at -20°C.

7.3.6 Running buffer

10X running buffer	
Reagent	**Weight**
Glycine	144g
Trizma® base (Sigma-Aldrich, St. Louis, MO, USA; 93349)	30.2g
SDS (Sigma-Aldrich, St. Louis, MO, USA; 71727)	8g
Water	Complete volume to 1L

Table 7.3-6 10X running buffer
The table above describes the composition of the 10X stock solution of running buffer. This solution can be stored at RT and ultrapure water should be used. Prior to use solution should be diluted to 1X with ultrapure water.

7.3.7 Transfer buffer

10X transfer buffer (Stock solution)*	
Reagent	**Weight**
Glycine (Sigma-Aldrich, St. Louis, MO, USA; G8898)	144g
Trizma® base	30.2g
Water	Complete volume to 1L
1X transfer buffer **	
Reagent	**Volume**
10X transfer buffer	150mL
Methanol (Fisher Scientific International, Inc., Hampton, NH, USA; A4124)	300mL
Water	1050mL

Table 7.3-7 Transfer buffer
This table describes the composition of the 10X stock solution and 1X transfer buffer solution. Only ultrapure water was used. *This stock solution can be stored at RT. **Prior to use the 10X stock solution was diluted to 1X, as described in the table.

7.3.8 Tris-buffered saline

10X TBS	
Reagent	**Weight**
Trizma® base	60g
NaCl	87g
Water	Complete volume to 1L

Table 7.3-8 10X TBS
The table above describes the composition of the 10X stock solution of TBS. This solution can be stored at RT, ultrapure water should be used and pH of the solution should be adjusted to 7.5. Prior to use solution should be diluted to 1X with ultrapure water.

7.4 Primary antibodies

Protein	Manufacturer (product number)	Dilution	Secondary antibody dilution
αCaMKII	Chemicon (MAB8699)	1:100,000	1:30,000
CREB**	Cell Signaling (9104)	1:1000	1:2000
GluA1	Millipore (05-855R)	1:1000	1:2000
pc-Jun	Santa Cruz (sc-16312-R)	1:200	1:2000
NSE	Millipore (AB951)	1:60,000	1:20,000
pαCaMKII	Abcam (ab5683)	1:1000 / 1:10,000*	1:2000
pCREB**	Cell Signaling (9191)	1:1000	1:2000
pGluA1	Millipore (04-823)	1:1000	1:2000
pRPT6**	Custume design by ProSci Inc., Poway, CA, USA	1:1000	1:20,000
Rpt6**	Enzo (BML-PW9265)	1:500	1:2000
Shank	Millipore (MABN24)	1:1000	1:2000
Synaptotagmin	Sigma (S2177)	1:30,000	1:2000
Ubiquitin	Millipore (05-1307)	1:1000	1:2000
Zif268	Santa Cruz (sc-110)	1:200	1:2000
c-fos	Abcam (ab7963)	1:500	1:2000
Lamin B1	Abcam (ab133741)	1:10,000	1:1000

Table 7.4-1 Primary antibodies

Table above shows the primary antibodies used in this thesis. Protein targeted by the antibody, manufacturer, dilution of the primary and secondary antibodies are presented on the table. *For samples from fraction S2 a dilution of 1:1000 was used. For samples from P2 fraction 1:10,000 dilution was used. **For good visualisation of these proteins 50μg of total protein *per* well was necessary.

7.5 Example images of western blot membranes

Images bellow are example of photos taken by chemioluminescent detection of different antibodies. Only images for molecules that presented signficitant alterations, and their respective internal control, are presented in this section.

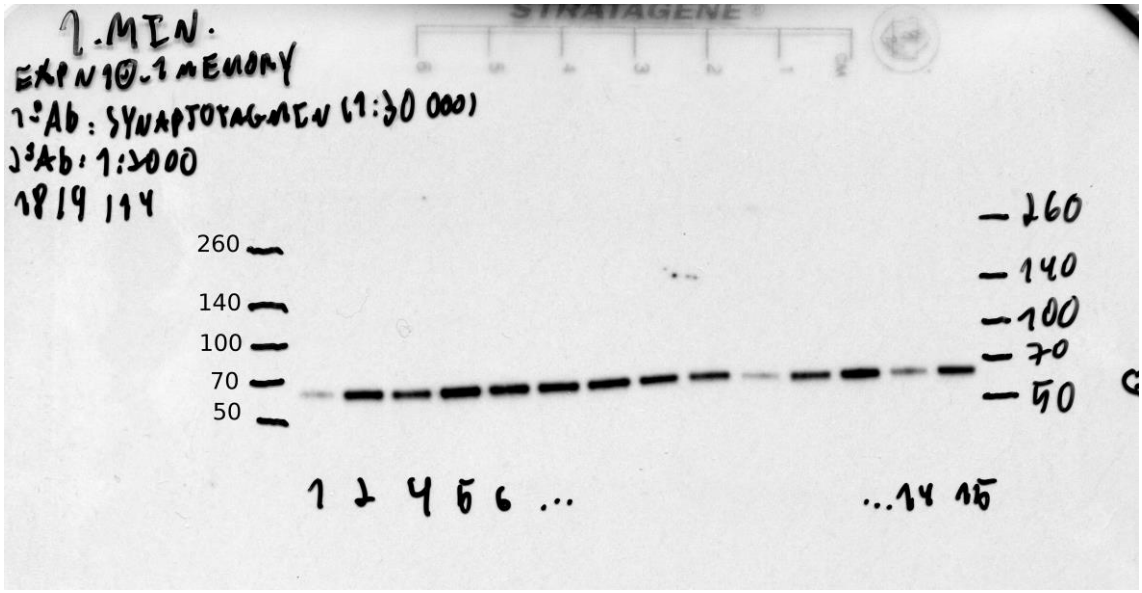

Figure 7.5-1 Example membrane of Synaptotagmin
The image above shows an example photo of a membrane tagged for Synaptotagmin. The unique band presented after antibody treatment was quantified. Synaptotagmin was used as internal control for samples from P2 fraction. Standers of different molecular weights can be seen on the first and last lane of the membrane.

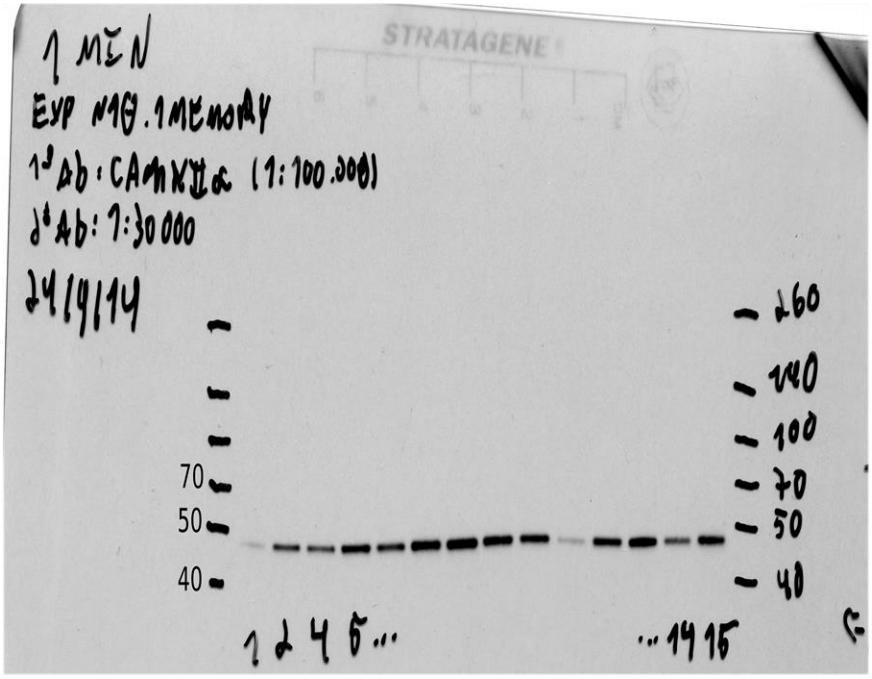

Figure 7.5-2 Example membrane of αCaMKII
This figure shows an example photo of a membrane tagged for αCaMKII. The unique band presented after antibody treatment was quantified. Although αCaMKII levels were not significant changed by treatment, αCaMKII levels were also used as internal control for the analyses of T286 αCaMKII phosphorylation. Standers of different molecular weights can be seen on the first and last lane of the membrane.

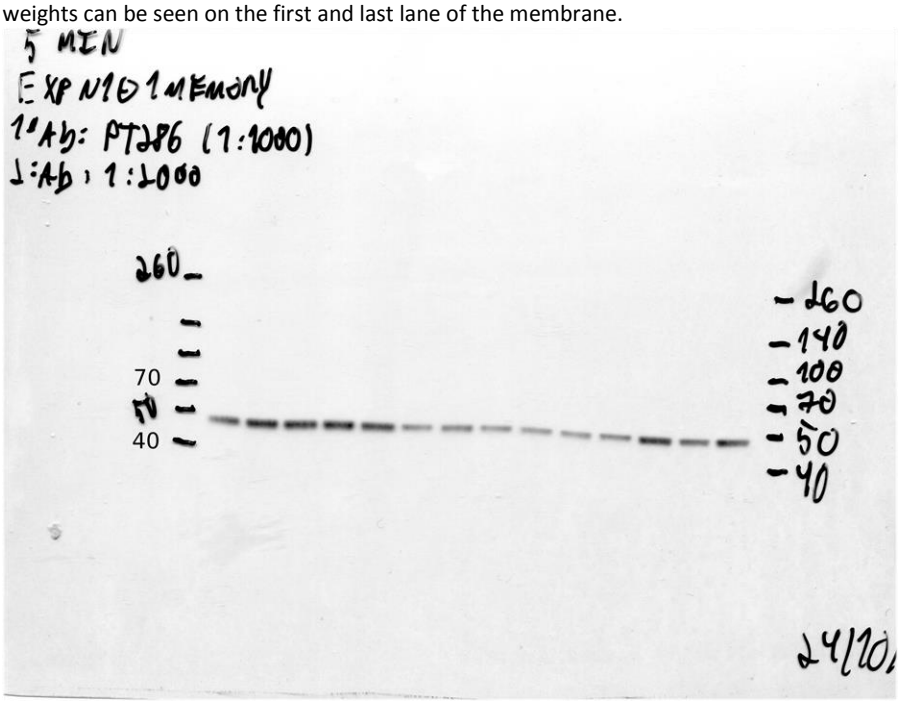

Figure 7.5-3 Example membrane of pCaMKII (T286)
This figure shows an example photo of a membrane tagged for T286 phosphorylation of αCaMKII. The unique band presented after antibody treatment was quantified. Standers of different molecular weights can be seen on the first and last lane of the membrane.

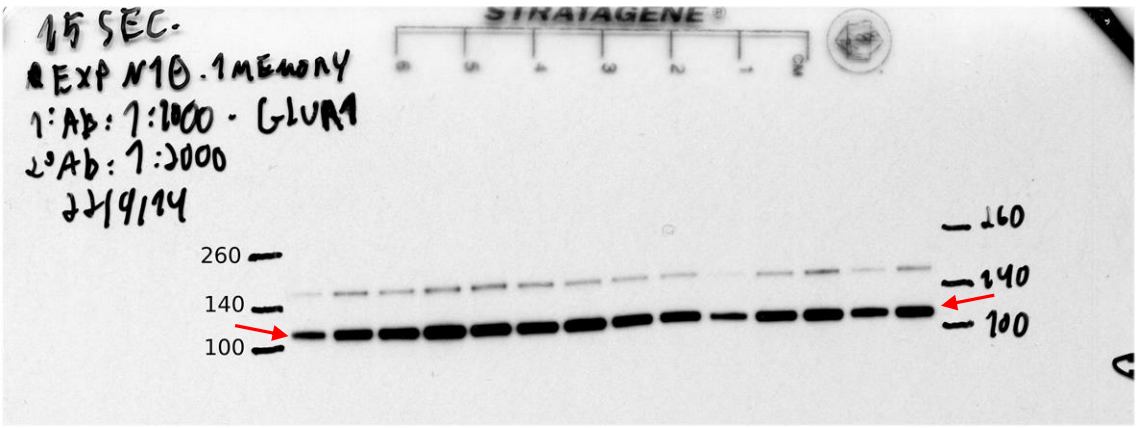

Figure 7.5-4 Example membrane of GluA1

This figure shows an example photo of a membrane tagged for GluA1. The band pointed by the red arrows was quantified. Standers of different molecular weights can be seen on the first and last lane of the membrane.

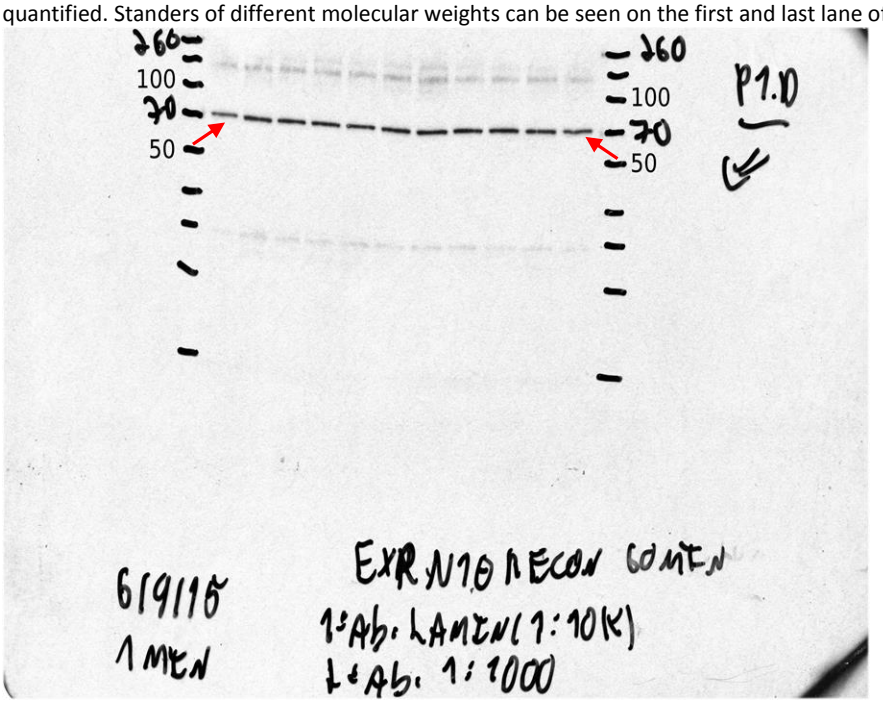

Figure 7.5-5 Example membrane of Lamin B1

This figure shows an example photo of a membrane tagged for Lamin B1. The band pointed by the red arrows was quantified. Lamin B1 was used as internal control for analysis of P1 samples. Standers of different molecular weights can be seen on the first and last lane of the membrane.

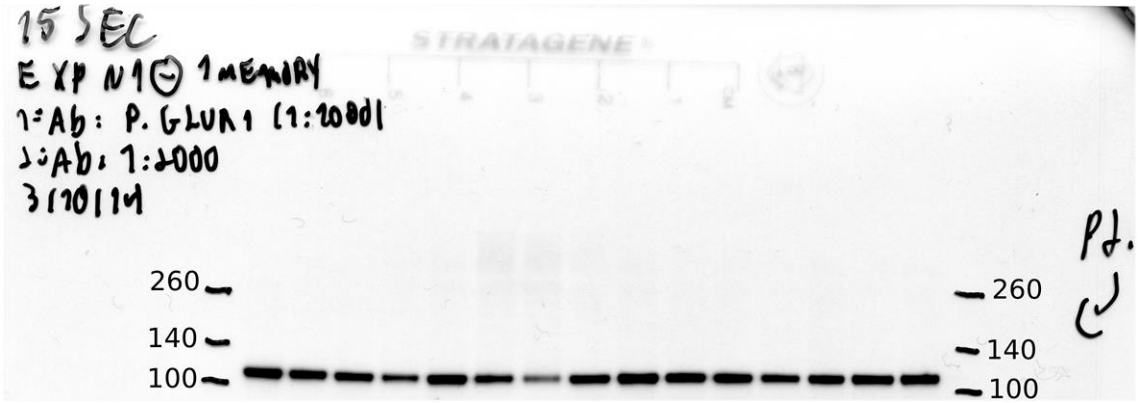

Figure 7.5-6 Example membrane of pGluA1 (S831)
This figure shows an example photo of a membrane tagged for S831 phosphorylation of GluA1. The unique band presented after antibody treatment was quantified. Standers of different molecular weights can be seen on the first and last lane of the membrane.

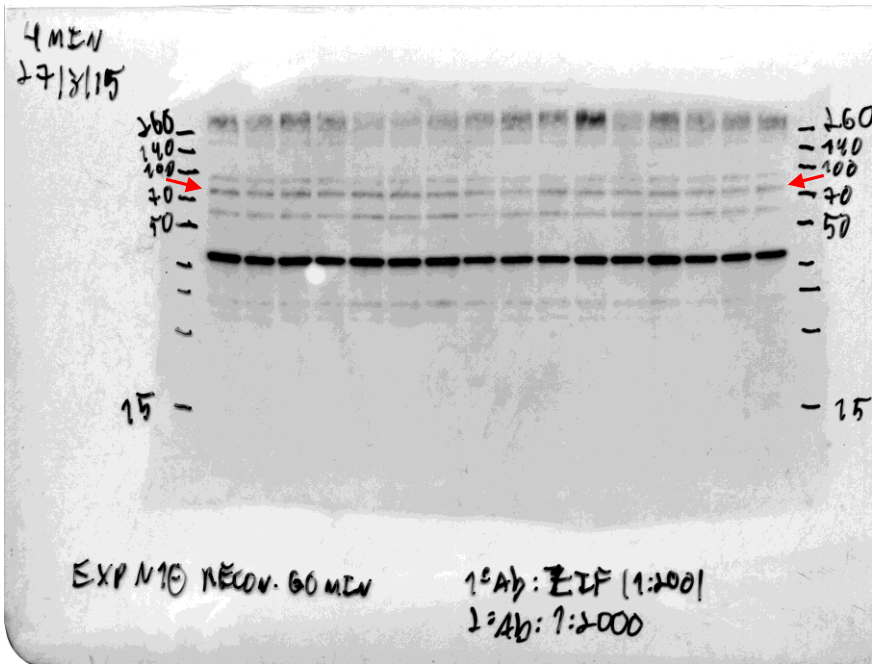

Figure 7.5-7 Example membrane of Zif268
This figure shows an example photo of a membrane tagged for Zif268. The band pointed by the red arrows was quantified. Standers of different molecular weights can be seen on the first and last lane of the membrane.

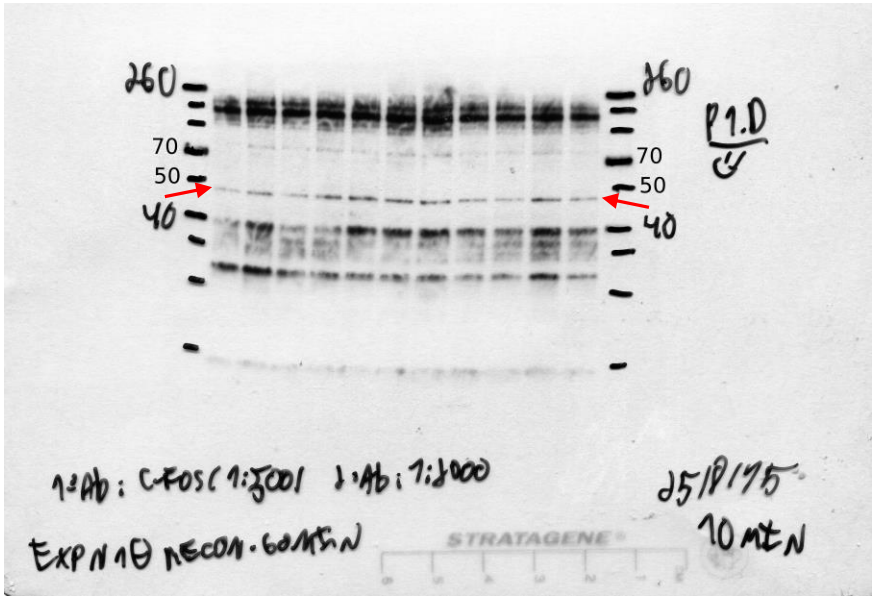

Figure 7.5-8 Example membrane of c-fos
This figure shows an example photo of a membrane tagged for c-fos. The band pointed by the red arrows was quantified. Standers of different molecular weights can be seen on the first and last lane of the membrane.

7.6 Failed experiment with CaMK2N2 knockdown

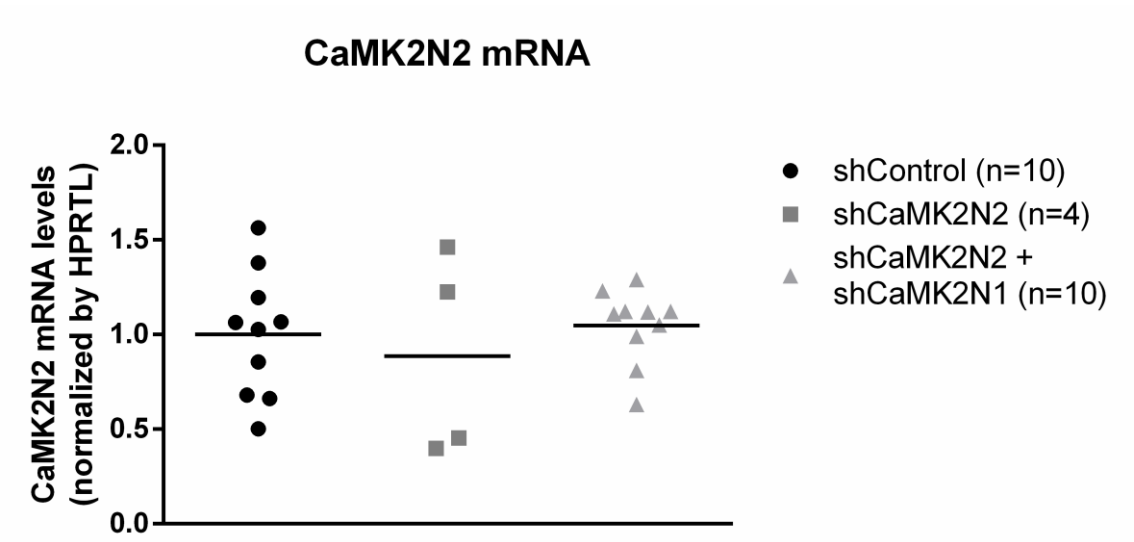

Figure 7.6-1 CaMK2N2 mRNA levels from experiment with CaMK2N2 knockdown
Quantitative analyses of the levels of CaMK2N2 mRNA, in dorsal hippocampus, 2 weeks after intra-hippocampal injection of viral vector solution. Groups are divided between control animals (shControl), animals that received injection of viral vector to knockdown CaMK2N2 (shCaMK2N2) and animals that receive injection of a combined solutions containing viral vectors to knockdown CaMK2N1 and N2 (shCaMK2N2 + shCaMK2N1). The result indicate that treatment with none of the viral vector solution had no effect in CaMK2N2 mRNA levels. CaMK2N2 mRNA levels were normalized by HPRTL mRNA levels. All results were standardize to shControl group average.

7.7 Failed experiment with CaMK2N1 overexpression

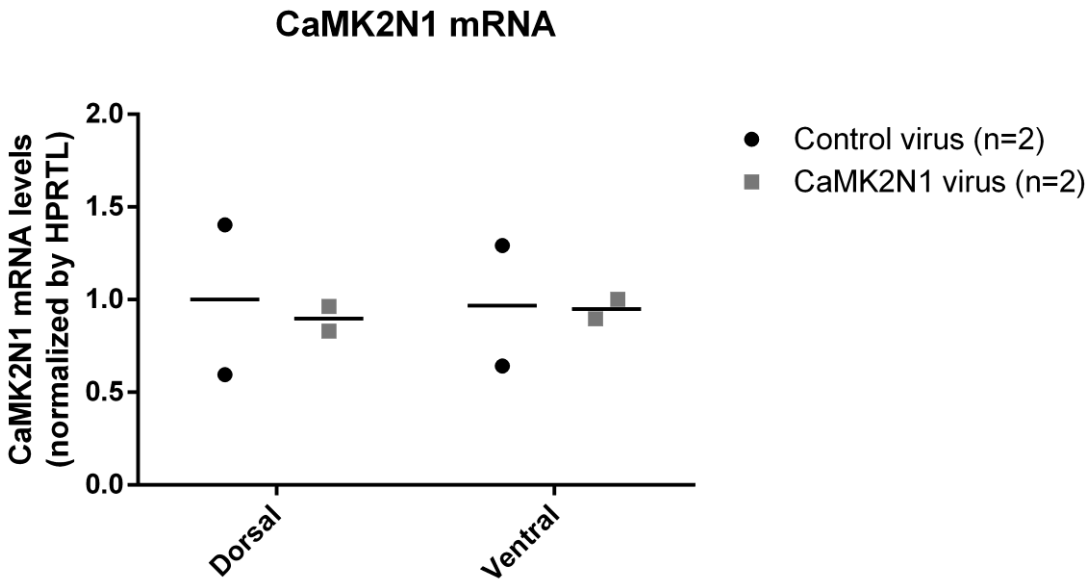

Figure 7.7-1 CaMK2N1 mRNA levels from experiment with CaMK2N1 overexpression
Quantitative analyses of the levels of CaMK2N1 mRNA, in dorsal hippocampus, 2 weeks after intra-hippocampal injection of viral vector solution to overexpress CaMK2N1. The result indicate that treatment with viral vector solution had no effect. CaMK2N1 mRNA levels were normalized by HPRTL mRNA levels. All results were standardize to Control virus group average.

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